University of Alberta

# Molecular identification and classification of phytoplasmas associated with aster yellows of different plant species

by

Persa Ceranic-Zagorac 

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To

*Tatiana and Nikola*

## Abstract

Phytoplasma-infected plants showing symptoms of aster yellows (AY) were derived from naturally infected symptomatic plants collected in the field such as spinach (*Spinacia oleracea* L.), pot marigold (*Calendula officinalis* L.), winged everlasting (*Ammobium alatum* L.), feverfew (*Chrysanthemum parthenium* (L.) Bernh.), and strawflower (*Helichrysum bracteatum* L.) or from a strain collection maintained by grafting in a greenhouse. Identification and classification of AY phytoplasma isolates in the last several decades were based primarily on vector relationships, host range and symptomatological characteristics. However, classification of phytoplasma isolates by symptomathology does not always coincide with phylogenetic relationships. In the present research, different molecular techniques were used to study genetic relatedness among phytoplasmas from various sources. Polymerase chain reaction (PCR)-based precedures using universal and specific primer pairs were employed effectively for detection and identification of 16 phytoplasma isolates. No PCR products were obtained from samples extracted from healthy plants. Further analysis on the genetic interrelationships among selected phytoplasma isolates were conducted by restriction fragment length polymorphism analyses. The work has resulted in the classification of newly collected isolates in the AY phytoplasma group. DNA heteroduplex mobility assay (HMA) and perpendicular temperature gradient gel electrophoresis analyses were used as additional molecular techniques in order to assess their sensitivity, reliability and simplicity for detection and differentiation of different phytoplasma isolates. Based on the results obtained in this research, HMA analysis, combined with PCR, has been shown to be a very accurate method for detection and differentiation of phytoplasmas.

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## List of abbrevations

| | |
|---|---|
| AY | aster yellows |
| BHAY | Belgian hydrangea aster yellows |
| CP | clover proliferation |
| CTAB | cetyltrimethylammonium bromide |
| DAY | dill aster yellows |
| EAY | eastern aster yellows |
| EDTA | ethylene diamine tetra acetic acid |
| FAY | feverfew aster yellows |
| FBP | faba bean phyllody |
| FHAY | French hydrangea aster yellows |
| FrAY | French aster yellows |
| HMA | heteroduplex mobility assay |
| PCR | polymerase chain reaction |
| RFLP | restriction fragment length polymorphism |
| PMAY | pot marigold aster yellows |
| PWB | potato witches'-broom |
| SAY | strawflower aster yellows |
| SpAY | spinach aster yellows |
| TBB | tomato big bud |
| TEMED | (N,N,N',N'- tetramethylethylenediamine) |
| Tm | melting temperature |
| TS | tomato stolbur |
| WAY | western aster yellows |
| WEAY | winged everlasting aster yellows |

# Introduction

Procaryotes, morphologically and ultrastucturally similar to animal mycoplasmas, were first observed in the sieve tubes of plants in 1967 (Doi *et al.*, 1967) and they were then called "mycoplasma-like organisms" (MLO). The plant-pathogenic MLOs, which are more appropriately renamed "phytoplasmas" (Sears and Kirkpatrick, 1994), have many characteristics in common with culturable members of the class Mollicutes. The phytoplasmas resemble animal mycoplasmas and spiroplasmas in that they lack cell walls and they can pass through a 0.45-µm-pore-size filter. A major difference between the plant and animal mollicutes is that the plant pathogens are found within phloem sieve tube elements, whereas most animal mycoplasmas grow between and among the host cells but not within them. Genetically and biochemically, phytoplasmas are not "mycoplasma-like"; at least four lines of genetic evidence support the conclusion that phytoplasmas are indeed members of the class Mollicutes (Sears and Kirkpatrick, 1994): **1)** the G+C genomic content of several phytoplasmas (25 to 30%) resembles the A+T-rich DNA values of culturable members of the class Mollicutes; **2)** the genetic phylogeny based on sequences from 16S rRNA genes of several phytoplasmas places them on the *Acholeplasma-Anaeroplasma* branch within the Mollicutes; **3)** because phytoplasma genome sizes vary from 450 to 1.180 Kb, the genome complexity is in the same range as that of many *Mycoplasma*

species, but the genome is substantially smaller than that of phytoplasmas phylogenetic relatives, the acholeplasmas and anaeroplasmas; 4) sequence data from several ribosomal protein genes confirm the phytoplasma-*Acholeplasma* phylogenetic relationship. On the basis of recent phylogenetic analysis of phytoplasmas and culturable members of the Mollicutes, the phytoplasmas most likely lost different functions than did mycoplasmas, spiroplasmas, ureaplasmas, and the newly created group entomoplasmas.

Phytoplasmas are very small wall-less procaryotes currently implicated as the causal agents of more than 300 different diseases among diverse plants, including fruit trees, forest and ornamental trees, grasses, vegetables, and flowers (McCoy *et al.*, 1989). For many years, phytoplasmal diseases of plants were considered virus diseases, based on such symptoms as vein-clearing, streaking, chlorosis, virescence of flowers, sterility, stunting, production of adventitous shoots, and abnormally erect growth. The pathogens were filterable, and some strains interfered with each other in plants and vectors. However, research into apparent viral diseases such as aster yellows found that, unlike most viral pathogens, the infectious agent could not be mechanically transmitted (Whitcomb and Black, 1982). The true nature of phytoplasmal agents of plant diseases remained undetermined largely because the agent could not be isolated or cultivated *in vitro*. Doi *et al.* (1967) and Ishiie *et al.* (1967) were the first to propose that phytoplasmas, a new group of fundamentally different disease agents, could be etiological agents of plant disease. Their evidence for phytoplasma etiology was based

on electron microscope observations of phytoplasmas associated with diseased but absent in healthy plants, and the concurrent disappearence of phytoplasmas and the remission of symptoms following treatments with tetracycline antibiotics. Since then, many supposedly viral plant diseases, which affect many important food, forage, and horticultural crops, have been reexamined and found to be probable phytoplasmal diseases (Maramorosch *et al.*, 1970; Whitcomb and Davis, 1970). Nearly all the reports of phytoplasmas have concerned yellow diseases whose causal agents are confined to phloem tissues (Maramorosch, 1974). Successful transmission of the disease agents was accomplished by grafting infected plant tissues to healthy plants, establishing vascular connection between healthy and diseased plants using parasitic dodder (*Cuscuta sp.*) or by allowing phloem feeding insects such as *Macrosteles fascifrons* (Stål) access to diseased and healthy plants (Kunkel, 1926; 1938; 1952).

Although Doi's historic observations (Doi *et al.*, 1967) established the existence of an entirely novel class of plant pathogens, phytoplasmas continue to be one of the most elusive groups of plant pathogens. The central difficulty in characterizing the vast majority of wall-less procaryotic plant pathogens has been the inability of any workers to culture them *in vitro* (Lee and Davis, 1986). For these and other reasons, basic research on phytoplasma-plant interactions has lagged far behind the detailed molecular analyses performed on other culturable plant pathogenic bacteria. However, the recent development of phytoplasma isolation procedures, which have allowed the production of phytoplasma-

specific antibodies (Caudwell *et al.*, 1982; Kirkpatrick and Garrott, 1984; Lin and Chen, 1985, 1986; Sinha, 1988) and the isolation and cloning of phytoplasma DNA (Kirkpatrick *et al.*, 1987; Davis *et al.*, 1987; Lee and Davis, 1988) has greatly facilitated taxonomical, etiological, and epidemiological studies on phytoplasmas. Cloned DNA probes have been applied in dot hybridizations and restriction fragment length polymorphism (RFLP) analyses (Deng and Hiruki, 1990b, 1991; Chen *et al.*, 1992; Lee et al., 1992; Davis *et al.*, 1993; Griffiths *et al*, 1994).

The recent introduction of polymerase chain reaction (PCR) assays has greately advanced the capacity to detect and identify phytoplasmas. PCR assays provide a very sensitive means of detecting a wide array of phytoplasmas by use of universal and specific primer pairs designed on the basis of phytoplasmas 16S rRNA gene sequence (Deng and Hiruki, 1990a, 1991a, 1991b; Ahrens and Seemüller, 1992; Davis and Lee, 1993; Lee *et al.*, 1993a, 1993b).

## Objectives

The objectives of this study were to: **1)** to determine if field collected material of various plant species showing phytoplasma-like symptoms were infected with phytoplasma and determine phytoplasma strain by using various molecular genetic techniques; **2)** compare different molecular genetic techniques in order to find out which one can be mostly recommended as a fast, sensitive, reliable and inexpensive method for detection and differentiation of various phytoplasma strains.

# Literature Review

## History of aster yellows

Aster yellows (AY) disease has been considered as a classic representative of yellows-type plant virus diseases since its first detailed description by Smith (1902). The disease has been described from North America, Europe, and Asia (Smith, 1902; Kunkel, 1926). The pioneering work of L.O. Kunkel at the Boyce Thompson Institute in the 1920's established that the agent of AY can infect mono- and dicotyledonous plants, belonging to some 40 families (Kunkel, 1926), in which it induces chlorosis, malformation, phyllody of flower, sterility, adventitous shoots, and stunting. Among economically important plants affected by the AY agent in nature are potato, onion, celery, carrot, lettuce, and spinach. The principal vector in North America is the six-spotted aster leafhopper, *Macrosteles fascifrons* (Stål) (Kunkel, 1926).

The agent of AY requires a long incubation in insect vectors and in plants. It can be inactivated in the body of the living vector and in certain living plants by heat treatments (Kunkel, 1941). Approximately 32°C was found to be critical temperature above which transmission by insects ceased.

Various naturally occurring strains of the AY agent have been recognized and their heat lability was found to differ (Granados and Chapman, 1968). As the result of cross protection strains of the AY agent can interfere with each other in inoculated plants and in vectors (Kunkel, 1955; Freitag, 1964). The agent has been filtered

through Berkefeld N and V filters (Black, 1943) but only with difficulty, and whenever the filtrate possessed infectivity the preparation was contaminated with a small bacterium, indicating that the agent was larger in size than known viruses. The agent has been transmitted to insects mechanically by needle inoculation (Black, 1940), but it has not been transmitted mechanically to plants. Serial passage from insect to insect provided direct evidence for its multiplication (Maramorosch, 1952). Cross protection between two strains of the AY agent has been described by Kunkel (1955) and interpreted as virus interference, or cross-immunity.

Attempts to find virus particles in AY-infected plants and insect vectors were unsuccessful. The agent was not purified by standard virus purification methods, including agar gel filtration (Steere, 1967) and differential centrifugation (Lee and Chiykowski, 1963). The riddle of its nature was finally understood in 1967, when Doi *et al.* (1967), Nasu *et al.* (1967) and Ishiie *et al.* (1967) suggested that AY and similar yellows-type diseases are caused by mycoplasma-like organisms.

Among the characteristic features that distinguish the AY agent from viruses are: morphology *in situ* in plant and in insect vector cells; susceptibility to cetain tetracycline antibiotics in plants and in insect vectors *in vivo*; degradative effect of relatively low temperature (below 47°C) on the agent in plants and in insect vectors *in vivo*. There are additional, indirect indications of pleomorphism of the agent, obtained by agar-gel filtration (Steere, 1967; Whitcomb and Davis, 1969) where infectivity was distributed in the column, whereas for a uniform virus particle a narrow,

concentrated zone would be expected. The induction of sterility in plants might also be a characteristic feature.

Many other aspects are common to both viruses and phytoplasma agents of plant diseases, which account for the initial difficulties in the identification of phytoplasma agents. The same species of insect vector may be able to transmit a virus and a phytoplasma. For instance, *M. fascifrons* can carry the oat blue dwarf virus (Zeyen and Banttari, 1969) as well as the AY phytoplasma agent. *Nephotettix sp.* can carry the rice dwarf virus as well as the phytoplasma agent of rice yellow dwarf. Deleterious effects on insect vectors may be caused by viruses, as well as by phytoplasmas, as in Western X phytoplasma (Jensen, 1959; Maramorosch and Jensen, 1963).

Further observations of phytoplasmas in AY-infected plants came from an electron microscopy study by Worley (1969,1970), who confirmed the presence of typical phytoplasma microorganisms in AY-diseased *N. rustica* and *C. chinensis.* Of particular interest were filamentous forms, beaded chains, and bodies that showed budding protrusions. These were similar to the phytoplasmas illustrated in 1967 in the sieve tubes of paulownia affected by witches'-broom (Doi *et al.*, 1967). Although these forms were interpreted as being in a presumptive propagative stage, they seem to represent stages of the degradation of phytoplasmas (Chen and Hiruki, 1977).

The observation that dodder *(Cuscuta sp.)* is often affected by yellows-type diseases when it acts as a bridge between diseased plants has resulted in several electron microscopy studies of dodder. The first observation of phytoplasmas in dodder, parasitizing AY-infected plants, was made by Dale and Kim (1969). The authors found

an abundance of phytoplasmas in dodder cells, which they interpreted as suggesting the multiplication of the microorganisms within the parasitic plant. They concluded that dodder is not a passive participant in the transmission of mycoplasmas, but that it plays the role of an alternate host.

**General properties of phytoplasmas**

Although phytoplasmas have been implicated as the causal agents of more then 300 woody and herbaceous plants (Lin and Chen, 1985; McCoy *et al.*, 1989), we know little about how many genetically distinct phytoplasmas cause these diseases. We know even less about the mechanism by which these pathogens cause disease. These pathogens require a dual host system comprising of alternating plant and arthropod hosts (McCoy, 1983). The pathogens are found in low and transitory concentrations within the phloem of affected plants but can ellicit profound disease symptoms (Sinha, 1983; Seemüller *et al.*, 1984). Phytoplasmas lack a cell wall; they are bounded by a triple-layered 'unit' membrane, and have cytoplasm, ribosomes, and both DNA and RNA. They are pleomorphic in shape due to the lack of a cell wall (Maramorosch and Raychaudhuri, 1981). Phytoplasmas are regularly observed within the sieve tube elements of plants and in the salivary glands of insect vectors (McCoy, 1983).

Electron micrographs of infected phloem tissues reveal numerous small vesicles with well defined trilaminar membranes approximately 7nm thick composed of two dense outer layers and a less dense intermediate layer (Sinha, 1976). Three dimensional images obtained by serial sectioning of infected plant tissues reveal

a complex of multibranched, beaded, filamentous or spheroid polymorphic bodies ranging from 175-400 nm in diameter for the spherical and oblong cells and up to 1700 nm long for the filamentous forms (Waters and Hunt, 1980; Florance and Cameron, 1978). Multiplication of phytoplasmas within the phloem apparently occurs, however, little is known about the mechanism. Phytoplasmas also multiply in the hemolimph, salivary glands, and intracellularly in various body organs of their insect vectors. Numerous attempts to culture phytoplasmas *in vitro* have been unsuccessful so far (Lee and Davis, 1986). Phytoplasmas are characterized by their resistance to penicillin (Whitcomb and Black, 1982) and by susceptibility to tetracycline-based antibiotics (Ishiie *et al.*, 1967; Davis *et al.*, 1968).

Among over 300 plant diseases associated with phytoplasmas, the most important are AY, apple proliferation, clover proliferation, coconut lethal yellowing, elm phloem necrosis, grapevine flevescence doree, papaya bunchy top, peach X-disease, peach yellows, pear decline, potato witches'-broom, mulberry dwarf, and rice yellow dwarf (McCoy *et al.*, 1989). Phytoplasma titers in infected plants are variable and in a state of dynamic change as shown by data of serological testing (Sinha, 1983) and nucleic acid hybridization (Kuske and Kirkpatrick, 1989; Deng and Hiruki, 1990b). Recently, significant progress, including detection, differentiation, and phylogenetic studies of phytoplasmas, has been made since the introduction of serological methods and recombinant DNA techniques to the study of phytoplasmas. The G+C content of several phytoplasma genomic DNA has been estimated ranging from 23.0 to

26.2% (Razin, 1985), by analyzing the hydrolyzed phytoplasma DNAs using high-performance liquid chromatography (HPLC) (Kollar and Seemüller, 1989). Phytoplasma genomic analysis has been also performed on a number of isolates using pulsed field electrophoresis (PFGE), field inversion gel electrophoresis (FIGE) (Neimark and Kirkpatrick, 1990; Davis *et al.*, 1990). Sequence data of 16S rRNA genes of three phytoplasmas indicated that phytoplasmas are evolutionarily most closely related to *Anaeroplasma* and *Acholeplasma* (Kirkpatrick and Fraser, 1989; Sears and Kirkpatrick, 1994). The estimated sizes of phytoplasma genomes examined range widely from 630 to 1185 kb (Davis *et al.*, 1990; Neimark and Kirkpatrick, 1991; Lim and Sears, 1991). Davis *et al.* (1988) were the first researchers to identify extrachromosomal DNA within the phytoplasmas. A large number of plasmids are now known to be associated with certain isolates of AY, virescence, phyllody, witches'-broom, maize bushy stunt, periwinkle little leaf, apple proliferation or elm yellows (Kuske and Kirkpatrick, 1990; Chen *et al.*, 1990; Kuske *et al.*, 1991; Denes and Sinha, 1992). It is expected that more detailed study of the genome of phytoplasmas will reveal the interrelationship between the gene structure and function. Employment of the new nucleic acid technologies to phytoplasma research has had a most significant impact on this important field.

## Symptoms and physiological changes induced by AY phytoplasmas

The term "yellows diseases" describes the most common symptoms observed in nearly all phytoplasma-infected plants.

However, in addition to chlorosis, there can be considerable variations in the other types of symptoms observed in phytoplasma-infected plants. Symptoms produced by one phytoplasma vary considerably in different plant hosts, and such differences in plant symptomatology have been historically used to define various phytoplasma "strains" (Freitag, 1964; Granados and Chapman, 1968). Changes in the morphology and physiology of infected plants can be divided into at least three main groups. The predominant symptoms caused by one group of phytoplasmas are general stunting and either a rapid or slow decline of the infected plant. Tree decline diseases such as X-disease, pear decline, coconut lethal yellowing, or citrus stubborn disease typify this group of pathogens. The loss of normal apical dominance, which results in the production of witches'-brooms (proliferations of branches, leaves, or floral parts) (Chang *et al.*, 1993), typifies the symptoms associated with the second group of plant pathogenic phytoplasmas. Proliferations are observed in phytoplasma diseases such as peach rosette (Knight, 1976), bunch disease of walnut (Seliskar, 1976), apple proliferation (Seemüller *et al.*, 1984), and maize bushy stunt as well as maize stunt disease caused by the culturable spiroplasma (Nault and Bradfute, 1979). Symptoms associated with the third major group of phytoplasmas include the production of leaf-like petals and sepals (phyllody) and the greening of normally pigmented floral parts (virescence). Enlargement and distortion of floral organs, such as that seen in AY disease and tomato big-bud disease, can also be caused by these virescence-inducing phytoplasmas. The conspicuous changes in plant morphology caused by these "virescence agents" were historically

considered to be caused by a single pathogen, the AY phytoplasma.

However, it is now recognized that other distinct virescence agents, such as the clover phyllody (Chiykowski, 1962), stolbur (Ploaie and Maramorosch, 1969; Garnier *et al.*, 1990), and the beet leafhopper virescence phytoplasma (Golino *et al.*, 1987) which differ from the AY phytoplasma in terms of their antigenicity (Lin and Chen, 1985), insect-vector relationships (Golino *et al.*, 1987), or morphological and physiological changes they induce in their plant hosts (Chiykowski, 1962, 1971; Golino *et al.*, 1988), can produce virescence and phyllody.

Although these phytoplasmas differ in some biological properties, the virescence and phyllody that they produce in a common hosts are usually indistinguishable. Like virus diseases, such disorders start with vein-clearing of leaves. The phloem-inhabiting phytoplasmas may, just like phloem-limited viruses (luteoviruses), cause phloem degeneration which then leads to atypical growth reduction, yellowing and premature death. Some diseases, like pear decline (psylla-transmitted) and elm phloem necrosis, are even exlusively characterized by deviations resulting from phloem necrosis. Many pathogenic phytoplasmas produce symptoms associated with more than one of these three symptoms groups.

It is quite possible that the genes which induce these types of alterations in normal plant development and morphology are similar in the various virescence phytoplasmas.

Phytoplasma colonization of phloem tissue can manifest itself by producing symptoms characteristic of a particular disease-host

combination (Markham and Towsend, 1979). Further information can be obtained from examining other properties such as host range and vector specificity (Chiykowski and Sinha, 1990; Freitag, 1967). Chiykowski and Sinha (1990) identified two categories of phytoplasma based on floral symptoms. Category 1 contains plants exhibiting phyllody and virescence of floral structures while category 2 contains phytoplasmas which cause reduction in floral size and color but never phyllody and virescence. Kirkpatrick (1991) identified three major groups, the decline agents, the proliferation agents, and the virescence agents. Differentiation within these groups is based on vector transmission species, host range, symptomatology and incubation period. Chiykowski and Sinha (1990) identified and segregated a number of Canadian phytoplasma diseases by vector transmission trials and symptomathology on diagnostic hosts. The study revealed that there are differences in efficiency between male and female vectors and between vector species. Pathogen transmission , therefore, appears to be a function of phytoplasma-vector specificity and plant species interaction. These parameters are of limited value since many of these traits may be controlled by a very limited number of genes (Kirkpatrick *et al.*, 1989). Phytoplasmas can have wide and overlapping host ranges and vector specificities. Symptoms produced by one phytoplasma may vary considerably in different plant hosts or different phytoplasmas may produce symptoms common to interactions between different combinations of hosts and pathogens, but thus they are not reliable for the characterization of the pathogen itself (Kuske *et at.*, 1991).

**Phytoplasma DNA isolation**

The yield of purified phytoplasmas is usually 2-4 mg per 100 g (dry weight) of leaves. This estimate is based on the yields from aster (*Callistephus chinensis* Nees) leaves affected by AY or clover phyllody, and celery (*Apium graveolans* L.) leaves affected by eastern peach-X disease (Sinha, 1979). The loss of phytoplasma during purification determined through serological titration was estimated to be about 75%. Various host plants infected with phytoplasmas may contain different concentrations of the pathogen, and therefore the yield of the purified phytoplasmas from various hosts can differ drastically (Sinha and Benhamou, 1983). The phytoplasma concentration may also vary in various parts of the host plant. ELISA studies condacted to determine the concentration of AY-phytoplasmas in various parts of the aster plants revealed that the highest phytoplasma-antigen concentration was in infected leaves (Arora and Sinha, 1988).

Phytoplasma DNA comprises not more than 0.5% of the total host plant DNA (Kollar et al., 1990). Isolation of highly purified plant DNA is notoriously difficult because of the formation of complexes of nucleic acids with secondary compounds, such as polysaccharides or polyphenols, released by cell disruption, leading either to the embedding of DNA in sticky geletinous matrix or to brown-colored products. Such DNA is neither restrictable nor suitable for PCR amplification. Phytoplasma DNA obtained from plants, additionally, will be also contaminated with fragments of plant DNA including chromosomal, mitochondrial and chloroplast DNA (Walbot and Goldberg, 1979). Denes and Sinha (1991) suggest that isolation of

phytoplasma DNA from plant roots is preferred because root tissue does not contain chloroplasts and flavenoids which could contaminate the extraction. The extraction of phytoplasma DNA for detection or cloning purposes generally have used partially purified intact phytoplasmas from phytoplasma enriched fractions obtained from enzyme-treated petioles or midribs of phytoplasma-infected plants (Lee and Davis, 1988).

Recently, highly purified phytoplasma DNA suitable for molecular cloning was obtained directly from crude genomic plant DNA preparations (Dellaporta *et al.*, 1983; Nigel *et al.*, 1991) or in combination with enzymatic digestion procedures (Sears *et al.*, 1989) which are then subjected to buoyant density separations (Kollar *et al.*, 1990), cesium chloride ethidium-bromide isopycnic centrifugation (Maniatis *et al.*, 1982) or equilibrium centrifugation of phytoplasma DNA utilizing bisbenzimide as a specific intercalating agent (Kollar and Seemüller, 1989). The buoyant density centrifugation techniques take adventage of the fact that phytoplasma genomes are rich in A-T sequences while host plant DNA have a higher G-C content than phytoplasma DNA. The bisbenzimide intercalating agents binds preferentially to A-T rich regions which reduces their buoyant density and facilitates their separation during ultracentrifugation.

Different extraction procedures has been used for crude nucleic acids preparation. DNA can be isolated from fresh or frozen material without pretreatment of tissue (Walbot and Goldberg, 1979; Murray and Thompson, 1980; Kollar and Seemüller, 1989). DNA can be purified from sieve elements that had been isolated by enzyme

digestion of veinal tissue from diseased plants (Lee and Davis, 1988). If mature sieve-elements from phytoplasma-infected plants are chosen as the original source for purifying phytoplasma DNA, there will be less host DNA such as DNAs of nuclei, mitochondria and chloroplasts. This is due to the fact that phytoplasmas are predominantly restricted to phloem tissue, i.e., sieve cells and sieve-tube elements.

Methods of DNA extraction without pretreatment are similar at the beginning of the procedure when plant material consisting mainly of vascular tissues is used. However, if cetyltrimethylammonium bromide (CTAB) procedure is used (Murray and Thompson, 1980), the plant powder is incubated with the extraction buffer. Phytoplasma DNA extraction from woody plants or plants with high content of secondary compounds requires modified method (Ahrens and Seemüller, 1992). Cell debris, proteins and polysaccharides are removed by repeated extraction with chloroform:isoamyl alcohol. Using the modified urea-phosphate-hydroxyapatite procedure (Walbot and Goldberg, 1979), tissue powder was incubated in the urea-containing buffer, and the lysate was extracted with chloroform : isoamyl alcohol. The DNA was purified on hydroxyapatite, eluted with a phosphate buffer gradient and recovered by high speed centrifugation.

## PCR amplification of phytoplasma 16S rRNA genes and RFLP analysis

### PCR: A historical perspective

The polymerase chain reaction (PCR) has revolutionized

molecular biology and its applications to nucleic acid research. PCR is an *in vitro* method that enzymatically amplifies specific DNA sequences using oligonucleotide primers that flank the region of interest in the target DNA. The principle involves a repetitive series of cycles each of which consists of template denaturation, primer annealing and extension of the annealed primers by a DNA polymerase to create the exponential accumulation of a specific fragment whose ends are determined by the 5' ends of the primers. The PCR is so named because it involves a polymerase and the products synthesized in each cycle can serve as templates in the next so the number of DNA copies approximatelly doubles at every cycle to create a chain reaction similar to the principles in a nuclear reaction. In practice the amplification is not a hundred percent efficient and 20 cycles will result in a $10^6$ to $10^8$ fold increase in PCR product. The concept of the polymerase chain reaction was first developed by Mullis (Mullis *et al.*, 1986; Mullis and Faloona, 1987) and was applied by a group in the Human Genetics Department at Cetus Laboratory to amplify human betaglobin DNA (Saiki *et al.*, 1985).

Initially PCR used the Klenow fragment of *E. coli* DNA polymerase 1 but this enzyme is inactivated by the high temperature needed for DNA denaturation at the beginning of each cycle, so fresh enzyme had to be added during each cycle. The different temperatures for each part of the cycle were obtained by moving the reactions to different water baths at the relevant temperatures.

The introduction of a thermostable DNA polymerase, *Taq* polymerase, first isolated from *Thermus aqquaticus* enabled the

amplification reaction to be carried out by cycling the temperature within the reaction tube after mixing all the reaction components (Saiki *et al.*, 1988). *T. aquaticus* strain Y T 1 is a thermophilic bacterium capable of growth at 70° to 75°C. It was originally isolated from a hot spring in the Yellow Stone National Park, USA, and described first in 1969 (Brock and Freeze, 1969). The optimum temperature for DNA synthesis is between 75° and 80°C and the enzyme can incorporate about 150 nucleotides per second per enzyme molecule. The introduction of *Taq* polymerase not only simplifies the procedure of PCR but also increases the efficiency and product yield because the higher optimum temperature allows the use of higher temperatures for primer annealing and extension, thereby increasing the overall stringency of the reaction. At 37°C which is the optimum temperature for the Klenow enzyme, non specific annealing can occur, resulting in nonspecific amplification products. *Taq* polymerase also enables the amplification of much longer fragments (up to 10 Kb) although these are amplified with reduced efficiency (Jeffreys *et al.*, 1988). The Klenow enzyme only enabled amplification up to about 400 base pairs.

In the last few years, the important advances in PCR have been modifications of the technique to enable amplification of DNA from archival material (Impraim *et al.*, 1987) making large data banks of paraffin embedded material available for study; modifications of the technique also have enabled PCR to be performed from single cells exploiting the technique's unique capability of massive amplification (Spann *et al.*, 1991).

**PCR and RFLP in diagnosis of phytoplasma infection**

Studies of highly conserved 16S rRNA sequences of the class Mollicutes have improved our understanding of their origin and phylogenetic relationships (Frydenberg and Christiansen, 1985; Taschke *et al.*, 1987; Woese, 1987). The advent of PCR in the late 1980's devalued all the previously developed DNA probes and kits, because PCR tests are more sensitive than those based on direct hybridization with a DNA probe. Moreover, PCR is fast, copying a single DNA sequence over a billion times in a few hours. Since the first applications of PCR to mycoplasma diagnose (Bernet *et al.*, 1989; Jensen *et al.*, 1989), the number of publications on PCR as a tool for Mollicutes diagnostics has been rising at an exponential rate like the PCR reaction itself.

PCR amplification of approximately 1-kb DNA sequences of 16S rRNA genes from several species of the genus *Mycoplasma* has been reported for the detection and identification of several mycoplasmas, including the human parasites *Mycoplasma salivarum*, *M. orale*, *M. genitalium*, and *M. hominis* (Blanchard *et al.*, 1991). PCR-RFLP techniques should be capable of detecting not only Mollicutes 16S rRNA genes in cell culture or its components, but also 16 S rRNA genes from Mollicutes species associated with human, animal, plant and insect diseases, and of predicting the phylogenetic relationships of these fascinating microorganisms (Deng *et al.*, 1992).

PCR methodology is still at a phase of rapid development and new procedures, modifications etc. appear almost daily. None of the technical aspects appear to be unique to phytoplasma PCR. Recently, the PCR reaction has been adopted for the detection and

classification of phytoplasmas. The first work concerning this technique was carried out utilizing 16S rRNA gene as this gene was the most widely studied (Deng and Hiruki,1990a, 1991a; Ahrens and Seemüller, 1992; Schaff *et al.*, 1992). Using PCR, detection sensitivity was increased by a factor of at least $10^2$ to $10^5$ fold over the previously used dot-blot hybridization methods. The detection sensitivity is in the following order: PCR > biotinylated riboprobe > biotinylated or radioactive dsDNA probe > radioactive oligonucleotide probe (Deng, 1991).

When phytoplasma DNA was not subjected to PCR, a minimum of 2.5 ng nucleic acid sample was needed to detect CP phytoplasma, whereas only $2.5 \times 10^{-2}$ to $2.5 \times 10^{-5}$ ng of nucleic acid was needed to detect CP phytoplasma DNA in periwinkle when PCR was employed. The primer design greatly affects the PCR amplification and consequently affects the detection sensitivity of phytoplasma. The primer pair with a high A+T content worked better than the one with a lower A+T content (Deng and Hiruki, 1990a).

Five PCR primer pairs were synthesized on the basis of the aligned 16 S-like rRNA gene sequences of eukaryotes and 16S rRNA gene sequences of eubacteria, Mollicutes, and intracellular organelles. These PCR primer pairs had high sequence homology to the conserved 16S rRNA genes of various culturable and nonculturable Mollicutes, but less sequence homology to the eukaryotic nuclear 16S-like rRNA or 16S rRNA genes of intracellular organelles. Full-length 16S rRNA genes and partial-length 16S rRNA genes of evolutionarily variable regions were successfully amplified when DNA preparations from culturable Mollicutes such as

*Mycoplasma flocculare* , three *Spiroplasma* strains and nonculturable Mollicutes associated with various plant diseases were used successfully as PCR templates. Amplification was not detected when *Escherichia coli* genomic DNA and DNA preparations from healthy plants were used under high stringency annealing conditions in thermocycling (Deng and Hiruki, 1991a).

Fifteen phytoplasma strains from North America and Europe, including AY, tomato big bud (BB), clover phyllody (CPh), chrysanthemum yellows (CY), and unknown phytoplasmas were studied by Lee *et al.* (1992). These phytoplasmas are among those previously identified, on the basis of dot hybridizations, as members of the AY phytoplasma strain cluster. RFLP analyses revealed that all 15 strains can be classified into three genomic types: type I (typified by eastern AY phytoplasmas), type II (e.g., western AY and CY phytoplasmas), and type III (e.g., CPh phytoplasma). Four key DNA probes (pAY9N, pBB88, pBB101, and pCN1-25) were designated for use in RFLP analyses for the identification of strains in the AY phytoplasma strain cluster and the differentiation of the types within this cluster. This classification is consistent with results from monoclonal antibody typing and polymerase chain reaction, but not with classification based on biological properties (Lee *et al*, 1992).

Lee *et al.* (1993b) developed a PCR-based procedure for classification and identification of phytoplasmas. A 'universal' primer pair was designed for specific amplification of 16S rDNA sequences from a broad array of phytoplasmas from infected plant tissues and RFLP analysis of the amplified 16S rDNA was used as the

basis for differentiation and classification of these uncultured phytoplasmas. Forty phytoplasma strains were used in this study and it was demonstrated that a PCR-based procedure using a "universal" primer pair can be employed effectively to detect and identify a broad array of known and unknown phytoplasmas from various plant hosts. By employing this primer pair in PCR, phytoplasma-specific 16S rDNA sequences were first amplified from nucleic acid samples extracted from all 40 phytoplasma-infected plants. No PCR products were obtained from samples extracted from healthy plants. Differentiation among and identification of these various phytoplasmas were achieved by subsequent RFLP analyses of the amplified phytoplasma 16S rDNA sequences by using a number of restriction enzymes. Based on similarity coefficient (F) derived from RFLP analyses, the 40 phytoplasma isolates analysed were differentiated and classified into nine distinct 16S rDNA groups and 14 subgroups. Of the nine groups delineated, groups 16SrI, 16SrIII, 16SrV, 16SrVI, and 16SrVII coincided with the phytoplasma strain clusters AY phytoplasma, peach X-disease phytoplasma, elm yellows (EY) phytoplasma, CP phytoplasma, and ash yellows (AshY) phytoplasma, respectively, which were delineated previously on the basis of DNA sequence homology by nucleic acid hybridization assays using randomly cloned chromosomal DNA fragments (Lee and Davis, 1992).

Paulownia witches' broom (PaWB) is one of the first plant diseases reported to be caused by phytoplasma (Doi *et al.*, 1967). PWB is prevalent in Japan, China, Korea and Taiwan and is a serious problem for the paulownia trees [*Paulownia tomentosa* (Thunb.)

Steud.], one of the best timber species in the world. The disease is generally characterized by the proliferation of branches with small yellowish leaves, followed by die-back of branches (Tsai *et al.*, 1988). On the basis of the nucleotide sequence of 16S rRNA genes, Namba *et al.* (1993) reported that Japanese phytoplasmas fall into three groups (I, II and III) and PaWB phytoplasma belongs to group I with onion yellows, tomato yellows and mulberry dwarf phytoplasmas. Using the same approach, Lee *et al.* (1994) reported that PaWB from Taiwan belongs to group I-B together with phytoplasmas of peach yellow leaf roll aand clover yellow edge.

Amplification of genes coding for ribosomal protein and 16S rRNA by PCR provides sensitive detection methods for PWB phytoplasma (Yoshikawa *et al.*, 1994). Based on the use of two primer pairs, disease-specific DNAs were detected after amplification of 100 pg of total DNA from infected leaves. The nucleotide sequences of amplified ribosomal protein and 16S rRNA genes showed that PWB phytoplasma is closely related to AY type phytoplasmas.

Ahrens and Seemüller (1992) developed a PCR method with a 558-bp fragment of the 16S rRNA gene as a template DNA and two oligonucleotide primers from conserved regions of this gene. The suitability of the system has been tested with 17 isolates of phytoplasmas maintained in periwinkle [*Catharanthus roseus* (L.) CT. Don] and with nine phytoplasmas samples from field grown woody plants. With DNA preparations enriched in phytoplasma DNA, an amplification product was obtained after 24 cycles. According to the restriction profiles, four distinct groups could be differentiated

among the phytoplasma examined. With the PCR system developed, a phytoplasma fragment was detected after amplification of approximately 18 pg of DNA from diseased periwinkle and 170 pg of DNA from infected woody plants.

PCR and restriction analyses of PCR-amplified DNA were also used to detect and differentiate strains of phytoplasmas associated with grapevine yellows detected in naturally diseased grapevines in the United States and Italy. At least three major groups of grapevine-infected phytoplasmas were delineated (Prince *et al.*, 1993).

CY phytoplasma in *Chrysanthemum* and phytoplasmas associated with 'germs fins' of *Gladiolus*, virescence of *Hydrangea,* witches' broom of *Brassica* and phyllody of *Ranunculus* have been detected by nucleic acid dot hybridization (Bertaccini *et al.*, 1990). Since concentrations of phytoplasmas in naturally-infected host plants may be low in many cases, pathogen detection methods giving greater sensitivity than that obtained with dot hybridization were required. A PCR protocol, previously designed for amplification of a DNA fragments from AY phytoplasma, was employed for the detection of phytoplasma DNA in field-collected and *in vitro* micropropagated plants. PCR with template DNA extracted from symptomatic, naturally-infected samples of *Brassica*, *Chrysanthemum* and *Hydrangea,* each yielded a DNA band corresponding to 1.0 Kbp. However, no DNA product was observed when either infected *Ranunculus* or *Gladiolus* was used as source of template nucleic acid for PCR; further experiments indicated absence of target DNA in the case of *Ranunculus* and the presence of

substances in *Gladiolus* which inhibited the PCR. The phytoplasma-specific DNA was detected by PCR using less than 95 pg of total nucleic acid in the case of field-collected *Hydrangea* and less than 11.4 pg of nucleic acid in the case of field collected *Brassica*. The findings illustrate highly sensitive detection of phytoplasma in both field-grown and *in vitro* micropropagated infected plants (Bertaccini *et al.*, 1992).

EY (=elm phloem necrosis) was first described in 1938 in the United States (Swingle, 1938). Until 1970, EY seemed to be limited to midwestern states. However, in the next decade, EY killed American elms (*Ulmus americana* L.) and red elms (*U. rubra* Muhl.) in northeastern states, including New York, Pennsylvania, New Jersey, and Massachusetts. Recently, EY was found in the Niagara Peninsula in Canada (Matteoni and Sinclair, 1989). Several research groups have investigated the epidemiology and vector(s) of EY, but progress has been limited because of the lack of a rapid and specific pathogen-detection method. PCR using oligonucleotide primer pairs, derived on the basis of the nucleotide sequence of probe pEY11, provided means for sensitive detection of EY phytoplasmas in infected elm tissue and for differentiation among EY phytoplasma variants. Preliminary results indicated the existence of various strains of EY phytoplasmas in North America that were distinct from a strain of EY phytoplasma present in Italy (Lee *et al.*, 1993a).

The mycoplasmal 16S rRNA genes carry in addition to the conserved regions more specific variable regions, as well as specific 16S-23S intergenic spacer regions (Harasawa *et al.*, 1993; Kirkpatrick *et al.*, 1994a, 1994b). Primers can be selected from

these regions with various degrees of specificity, ranging from clusters of species (Davis and Lee, 1993; Lee *et al.*, 1994), single species, down to the subspecies level (Robertson *et al.*, 1993). Recently, Firrao *et al.* (1993) have succeeded in preparing specific primers from 16S rRNA gene capable of detecting two phytoplasma diseases, namely clover phyllody and apple proliferation through PCR only.

**Heteroduplex mobility and tracking analysis (HMA)**

HMA is a new method developed for the detection and estimation of genetic divergence between different genotypes and their strains without costly and laborious large-scale DNA sequencing (Delwart *et al.*, 1993). This quantitative assay is a rapid, accurate and simple screening tool based on the observation that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Heteroduplexes, or DNA hybrids, are generated by base pairing between complementary single strands derived from the different parental duplex molecules; it occurs during genetic recombination. Unknown DNA sequences can be compared against themselves or standard reference sequences. The DNA sequence of genetically common and rare variants could, therefore, be determined on a selective rather than random basis.

Recently, the use of HMA has allowed simple and accurate classification of nine different phytoplasma isolates (Zhong and Hiruki, 1994).

**Temperature gradient gel electrophoresis (TGGE) analysis**

Gel electrophoresis is a widely used technique to separate biomolecules due to their different mobility in the gel. This mobility is determined by charge, size, and shape of the molecules. The shape, more generally regarded as the conformation of a molecule, may undergo structural transitions which are inducible by several parameters, such as temperature, concentration of denaturing agent, *etc.* An electrophoretic technique, which includes the variation of such a parameter, would be able to separate different molecules not only according to charge, size, and shape but also according to their conformational transitions or stability of certain conformation. Conformational transitions of interest may be induced by changes in temperature, in solvent conditions, or in the ligand concentration; pressure and electric field may induce transitions as well. So far, the most extensive theoretical treatment has been given for temperature-dependent structural transitions. TGGE method separates DNA molecules according to their thermal stability (Rosenbaum and Riesner, 1987; Ruzicka *et al.*, 1993). TGGE may be applied to analyze quite different problems in biochemistry and molecular biology of nucleic acids. If the temperature-gradient is established perpendicular to the electrophoresis, monomolecular conformational transitions of nucleic acids show up as continuous transition curves; strand-separation leads to discontinuous transitions (Riesner *et al.*, 1989). The gradient may be freely selected between 10° and 80°C, and is highly reproducible and linear. TGGE can be used in order to detect single base pair mutations in the open reading frame of a gene. Changes in a single base are

distinguishable by means of the analysis of the heteroduplexes, and changes in more than two bases can be distinguished in both homoduplexes and heteroduplexes (Barros et al., 1994). TGGE was also applied to separate homo- and heteroduplexes (Riesner *et al.*, 1992).

The temperature gradient can also be established parallel to the electric field. In that case the sample migrates in a normal narrow slot from low temperature to high temperature. At the temperature of partial denaturation, the nucleic acid is drastically retarded and nucleic acids of the same size but of different thermal stability can be separated. In such a parallel TGGE, as many samples as in normal polyacrylamide gel electrophoresis can be analyzed, but only the position of the band that is characteristic for the thermal stability of the nucleic acid (and not the whole transition curve) can be obtained (Riesner *et al.*, 1989).

# Materials and methods

## Phytoplasma isolates

The phytoplasmas included in this study were either obtained from periwinkle (*Catharanthus roseus* (L.) G. Don) as an experimental host or from their naturally infected host plants grown at the Crop Demonstration Plot of the Alberta Department of Agriculture and Rural Development, Edmonton, Alberta in 1994.

The following isolates were maintained in periwinkle: Alberta aster yellows (**AY27**), western aster yellows (**WAY**, Ottawa isolate), eastern aster yellows (**EAY**, a New York isolate), French aster yellows (**FrAY**), hydrangea aster yellows (**FHAY**, France; **BHAY**, Belgium), tomato stolbur (**TS**, France), potato witches'-broom (**PWB**, Alberta), clover proliferation (**CP**, Alberta), tomato big bud (**TBB**, Australia) and faba bean phyllody (**FBP**, Sudan).

PWB, CP, TBB and FBP were used as a negative control.

**EAY**, a New York isolate, was kindly provided by Dr. T.A. Chen, Rutgers University, NJ. **CP**, **PWB** (Chen and Hiruki, 1975; Hiruki and Chen, 1984), and **AY27** (a subculture of AP-1, Alberta isolate) were originally collected in Alberta (Chen and Hiruki, 1977, 1978). **FAY**, **FHAY**, **BHAY**, **FBP**, **TS** and **TBB** were kindly provided by M.T. Cousin, Station de Patologie végétale, France.

Field samples from naturally infected plant species showing symptoms of aster yellows were collected in the Edmonton area (Ceranic-Zagorac and Hiruki, 1994) from pot marigold (*Calendula officinalis* L.) (**PMAY**), winged everlasting (*Ammobium alatum* L.)

(**WEAY**), spinach (*Spinacia oleracea* L.) (**SpAY**), strawflower (*Helichrysum bracteatum* L.) (**SAY**), feverfew (*Chrysanthemum parthenium* (L.) Bernh) (**FAY**) and dill (*Anethum graveolens* L.) (**DAY**).

**DNA isolation**

A modified phytoplasma-enrichment procedure, described by Kirkpatrick *et al.* (1987), was used . Midribs (0.5 g) were cut into small pieces with a scissors, then incubated for 10 min in 6 ml of ice cold grinding buffer (125 mM potassium phosphate, 30 mM ascorbic acid, 10% sucrose, 0.15% bovine serum albumin [BSA], 2% polyvinylpyrrolidone [PVP-15], pH 7.6) in a mortar. After initial grinding, 8 ml of fresh buffer was added to the tissue, and grinding was repeated. The homogenate was centrifuged at 4°C for 4 min at 1,100 *g*. The supernatant was decanted and recentrifuged at 4°C for 25 min at 14,600 *g*. The phytoplasma-enriched pellet was resuspended in 1.5 ml of warm (60°C) extraction buffer acording to Doyle and Doyle (1990) (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) and was incubated at 60°C for 30 min. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). After centrifugation, the aqueous layer was precipitated with a two-third volume of -20°C isopropanol and was then centrifuged at 15, 000 *g* with a microfuge. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 100 µl of water.

**PCR primers**

The universal primer pair R16 F2/R2 designed on the basis of 16S rRNA sequence from a strain of Michigan AY phytoplasma (MIAY-86-7) (Lim and Sears, 1989) was used in this study. The oligonucleotide sequence of the forward and the reverse primers and base locations are:

**R16F2, 5'-ACGACTGCTGCTAAGACTGG-3'** (base 152-168);

**R16R2, 5'-TGACGGGCGGTGTGTACAAACCCCG-3'** (base 1373-97).

The universal primer pair was used in PCR assays for general detection of various phytoplasmas.

Phytoplasma 16S rRNA group-specific primer pair R16 F1/R1 was designed on the basis of unique sequence of phytoplasma 16S rDNA in the region (about 1.2 kb) amplified with the universal primer pair R16 F2/R2 (Lee *et al.*, 1994). The oligonucleotide sequences of this group-specific primer pair are:

**R16(I)F1, 5'-TAAAAGACCTAGCAATAGG-3';**

**R16(I)R1, 5'-CAATCCGAACTGAGACTGT-3'.**

**PCR amplification**

The DNA fragments were amplified from 10 µl of 2- to 5-fold diluted DNA samples extracted from phytoplasma-infected plants. The amplification was performed in 100 µl of PCR reaction mixture containing 200 µM each of dATP, dCTP, dGTP and dTTP, 1µl each of upstream and downstream primer (0.6-0.8 µM), 10 µl of 10x PCR reaction buffer and 2.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega Corp.), and 100 µl of mineral oil. Thirty PCR cycles were

conducted in an automated thermocycler. The following parameters were used: 1st cycle, denaturation 5 min at 94°C; annealing 2 min at 58°C; extention 3 min at 72°C; the next 28 cycle, denaturation 1 min at 94°C; the final cycle, extension for 7 min at 72°C. Denaturation and annealing conditions were the same as for the first cycle. To analyze undigested PCR amplification products obtained from DNA of healthy and infected plants, 10 µl of the reaction mixture were analyzed by electrophoresis in 1.0% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator.

**RFLP analyses of PCR products**

16S rDNA sequences were analyzed by restriction endonuclease digestion. Between 3 and 10 µl (100-200 ng DNA) of each PCR product was digested separately with 1 µl (10U) of undiluted *Alu* I or *Kpn* I restriction endonuclease (Promega Corp.) at 37°C for 2 h. Recognition site for *Alu* I is 5'...AG/CT...3', and *Kpn* I recognizes the sequence 5'...GGTAC/C...3'. The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel in 1x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) and stained in ethidium bromide. DNA bands were visualized using a UV transilluminator, and the RFLP patterns were then compared. Molecular weights were determined from fragments produced by the digestion of øX 174 DNA with *Hae* III.

**Heteroduplex mobility analysis (HMA)**

A sample of 10 µl of PCR products from each of the phytoplasma isolates was combined with 8 µl of AY27 PCR product, then 2 µl of 10x annealing buffer was added (1 M NaCl, 100 mM Tris-HCl [pH 7.8], 20 mM EDTA). DNA was denatured at 94°C for 2 min and renatured by rapid cooling on ice. The DNA fragments were separated in 5% polyacrylamide gel (29:1 acrylamide : bis) in 1x TBE buffer at 200 V for 3 h, followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. Heteroduplex mobilities were calculated as the average distance of migration of the homoduplex bands.

**Perpendicular temperature gradient gel electrophoresis (TGGE)**

Gels for TGGE contained 5% acrylamide, 0.12% bisacrylamide, 0,04% TEMED, in 0.1 x TBE buffer (8.9 mM Tris, 8.9 mM boric acid, 0.25 mM EDTA) and 0.07% ammonium peroxodisulfate. Polyacrylamide gels were prepared on GelBond films. For preparation the gel was poured between a glass plate and a GelBond film which was pressed onto a second glass plate. The spacers were 1 mm thick. After polymerization on the GelBond film, the gel was laid on a temperature-gradient plate.

Amplification products of AY27 and mixture of AY27 and PMAY and AY27 and TS (after a denaturation/renaturation cycle; conditions same as for HMA) were diluted in 0.1 x TBE containing bromophenol blue and Xylene Cyanol FF dye. The nucleic acids samples (about 300 ng in 180 µl) were applied to a broad sample slot, expanding over

nearly the whole width of a slab gel, and subjected to gel electrophoresis in 0.1 x TBE in DIAGEN-TGGE gel apparatus (DIAGEN GmbH, Germany). In the first step of perpendicular TGGE the nucleic acids samples were allowed to migrate into the gel matrix under a nondenaturing temperature of 10°C for 1.5 hr at 300 V. Subsequently, the right circulating water bath was heated to 65°C and the temperature of the left side remained 10°C. The gel was then run at 500 V for 2.5 hr, and silver stained as described by Rosenbaum and Riesner (1987).

# Results

## Symptoms in diseased plants

Symptoms on diseased periwinkle plants grown in the greenhouse and on symptomatic plants collected from the field included proliferation of axillary shoots resulting in a "witches'-broom" appearance, virescence, phyllody, severe stunting, vein-clearing, purple coloration and yellowing of the leaves (Figs. 1-4).

## Detection of phytoplasma 16S rRNA gene from plants by PCR

It was difficult to obtain total genomic DNA from symptomatic plants pure enough to be a template for PCR. The most problematic plants were those collected from the field. A modification of the phytoplasma-enrichment procedure described by Kirkpatrick *et al.* (1987) was used for isolation of highly purified genomic DNA (Fig. 5). Concentration of total DNA was established by spectrophotometric measurement and it was in the range from 50 ng/µl to 1.6 µg/µl.

Using the universal primer set, R16F2/R2, a 1.2 kb DNA fragment of the 16S rRNA gene was amplified in all 16 DNA samples extracted from infected plants. However, no DNA product was observed when symptomatic dill was used as source of template nucleic acid for PCR. Control reactions containing template DNA extracted from healthy periwinkle, pot marigold, spinach, hydrangea and strawflower yielded no observable DNA amplification. (Fig. 6).

The primer pair R16F1/R1 specifically initiated amplification of 16S rDNA sequences (1.1 kb) among phytoplasma strains in the

Fig.1. Healthy (h) and diseased (d) plants of periwinkle (A) and aster (B).

h
d
A

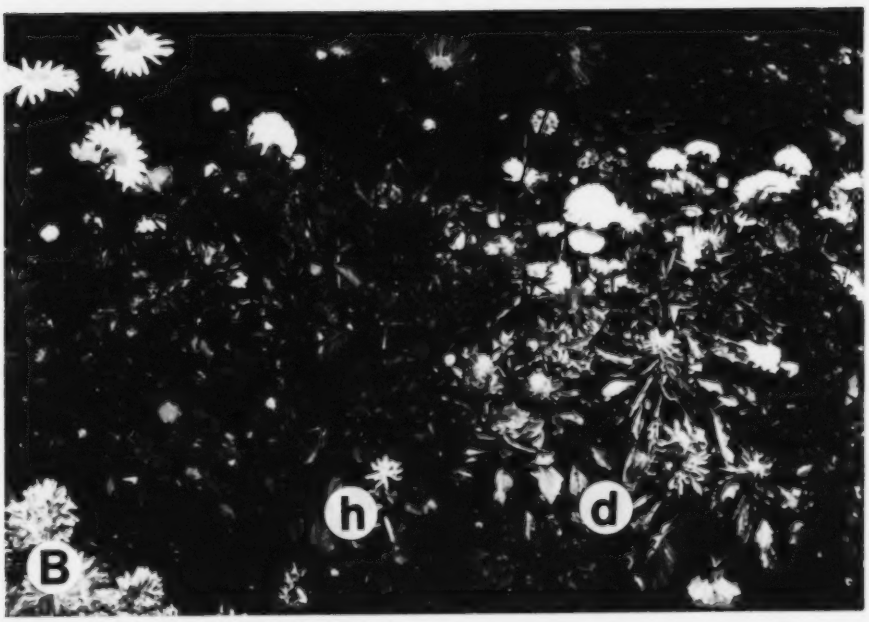

h
d
B

Fig. 2. Different host plants showing symptoms of aster yellows infection:

A) Aster;
B) Periwinkle;
C) Spinach;
D) Strawflower;
E) Pot marigold
F) Feverfew;
G) Hydrangea;
H) Winged everlasting (early symptoms);
I) Winged everlasting (late symptoms);
J) Dill.

A

B

C

D

E

F

G

H

I

J

Fig. 3. Floral symptoms on alsike clover infected with clover proliferation phytoplasma isolate.

Fig. 4. Potato infected with potato witches'-broom phytoplasma isolate.

3

4

Fig. 5. Genomic DNA isolated from plant hosts infected with different phytoplasma isolates. Concentration and purity of DNA differed from sample to sample. Lanes 1 and 19, lambda DNA digested with *Hind III*, from top to bottom band (kb): 23.1, 9.4, 6.6, 4.4, 2.3, 2.0. From lane 2 to 18: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, FAY, DAY, TS, PWB, CP, FBP and TBB.

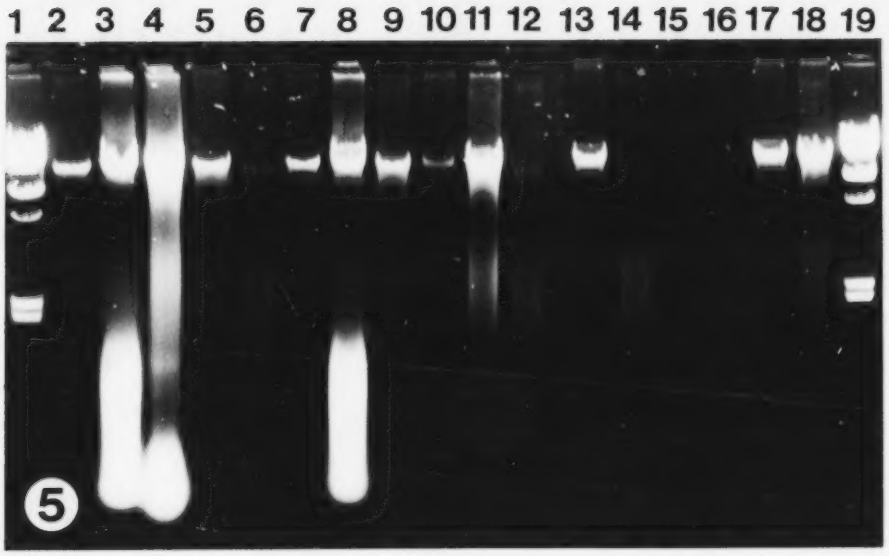
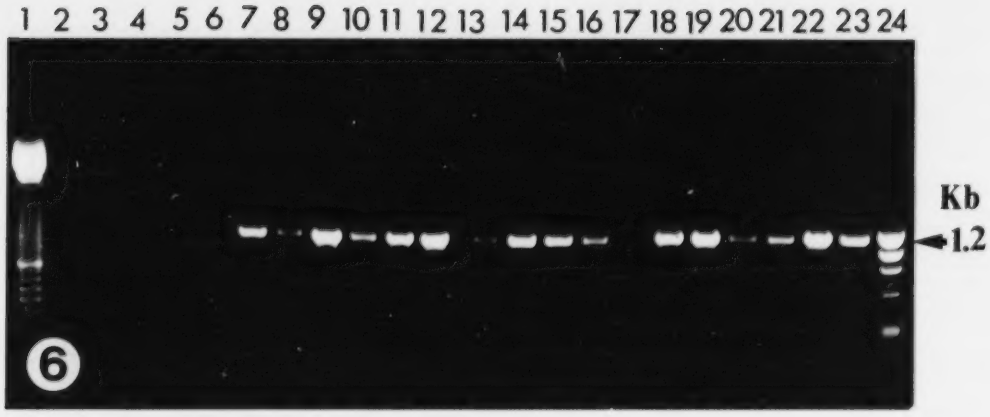

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
⑤

Fig. 6. PCR amplification of a 16S rDNA fragment from various phytoplasma isolates using the universal primer pair, R16F2/R2, separated by electrophoresis through a 1% agarose gel.Lane 1, 100 bp DNA ladder; lane 24, ØX174 RF I DNA *Hae* III digest, fragment sizes in base pairs from top to bottom are: 1.353, 1.078, 872, 603, 310. Lanes from 2 to 6, control from healthy periwinkle, hydrangea, pot marigold, spinach and strawflower. Lanes from 7 to 23 are: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, DAY, FAY, TS, PWB, CP, FBP and TBB.

Fig. 7. Analyses of phytoplasma DNAs amplified by PCR using the group-specific primer pair R16F1/R1. Lanes 1 and 18, 100 bp and ØX174 RF I DNA markers respectively; lane 2, healthy periwinkle; lanes from 3 to 17: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY SpAY, SAY, DAY, FAY, TS, FBP and TBB.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Kb
1.2
6

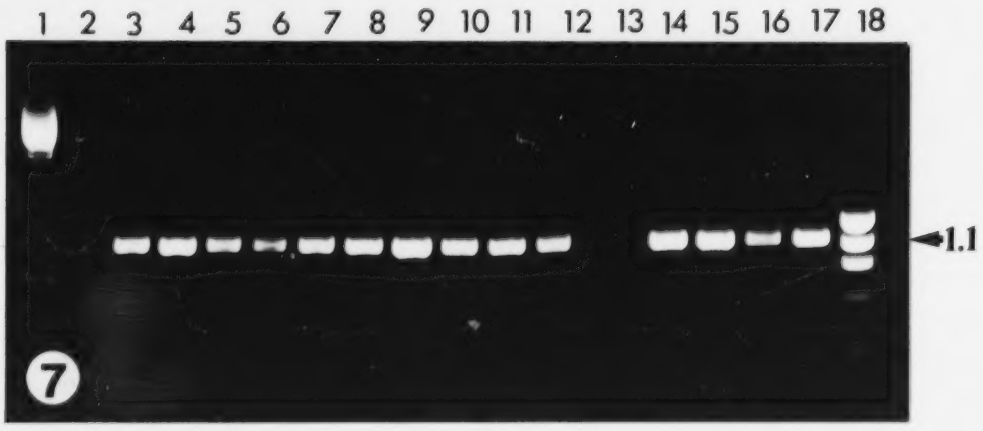

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
1.1
7

Table 1. Some characteristics of primers used for PCR amplification of a 16S rDNA fragment from various phytoplasma isolates.

| Primer | Sequence length | Tm (°C) | GC (%) |
|---|---|---|---|
| R16F1 | 19 | 51.0 | 36.8 |
| R16R1 | 19 | 55.4 | 47.3 |
| R16F2 | 20 | 59.8 | 55.0 |
| R16R2 | 25 | 68.5 | 64.0 |

Table 2. Summary results from (PCR) amplification of 16S rDNA from different phytoplasma isolates.

| Template DNA | 16S rDNA amplification[a] using primer pair | |
|---|---|---|
| | R16 F2/R2 | R16 F1/R1 |
| Phytoplasma isolate | | |
| AY27 | + | + |
| AYW | + | + |
| EAY | + | + |
| FrAY | + | + |
| FHAY | + | + |
| BHAY | + | + |
| PMAY | + | + |
| WEAY | + | + |
| SpAY | + | + |
| SAY | + | + |
| FAY | + | + |
| DAY | - | - |
| TS | + | + |
| FBP | + | + |
| TBB | + | + |
| CP | + | - |
| PWB | + | - |

[a]+= Specific PCR product, −= no detectable PCR products

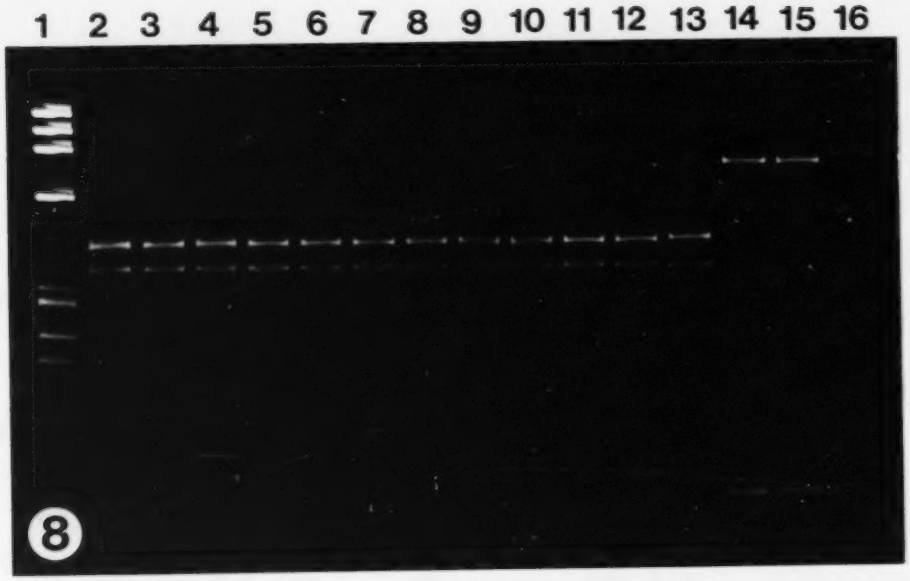
phytoplasma 16S rRNA group I, which includes AY phytoplasmas and related strains (Lee *et al.*, 1993b). There was no amplification of DNA sequences when nucleic acids isolated from CP and PWB phytoplasma infected plants were used as a templates for PCR amplification (Data not shown in Fig. 7). Some characteristics of primers used for amplification are given in Table 1, and summary of results from PCR amplification of 16S rDNA from 16 phytoplasma isolates are presented in Table 2.

**RFLP analyses of phytoplasma 16S rDNA sequences**

Phytoplasma 16S rDNA sequences were analyzed by separate digestion using two selected restriction enzymes, *Alu* I (Figs. 8 and 10) and *Kpn* I (Figs. 9 and 11). RFLPs obtained using this restriction enzymes had been found to distinquish the AY group from other phytoplasma groups (Lee *et al.*, 1993b). Analyses of the PCR products amplified with the universal primer pair R16F2/R2 resulted in three (Fig. 8) or two (Fig. 9) collective 16S rDNA RFLP pattern types. Collective RFLP patterns (based on analyses with *Alu* I and *Kpn* I) among the isolates AY27, WAY, EAY, FAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, FAY and TS were identical or very similar. TS had a slightly different pattern when digestion of 1.2-kb product was performed with *Alu* I (Fig. 8, lane 13). An identical RFLP pattern was observed between isolates PWB and CP (lane 14 and 15), previously designated as members of the CP phytoplasma isolate cluster (Lee *et al.*, 1991), which was completely different from other phytoplasma isolates. TBB (lane 16) gave a pattern different from all AY and CP-cluster isolates when restricted with *Alu* I only.

Fig. 8. Polyacrylamide gel electrophoresis of *Alu* I digest of phytoplasma 16S rDNA amplified by PCR using the primer pair R16F2/R2. Lane 1, øX174 RF I DNA marker; lanes from 2 to 16: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, FAY, TS, PWB, CP and TBB.

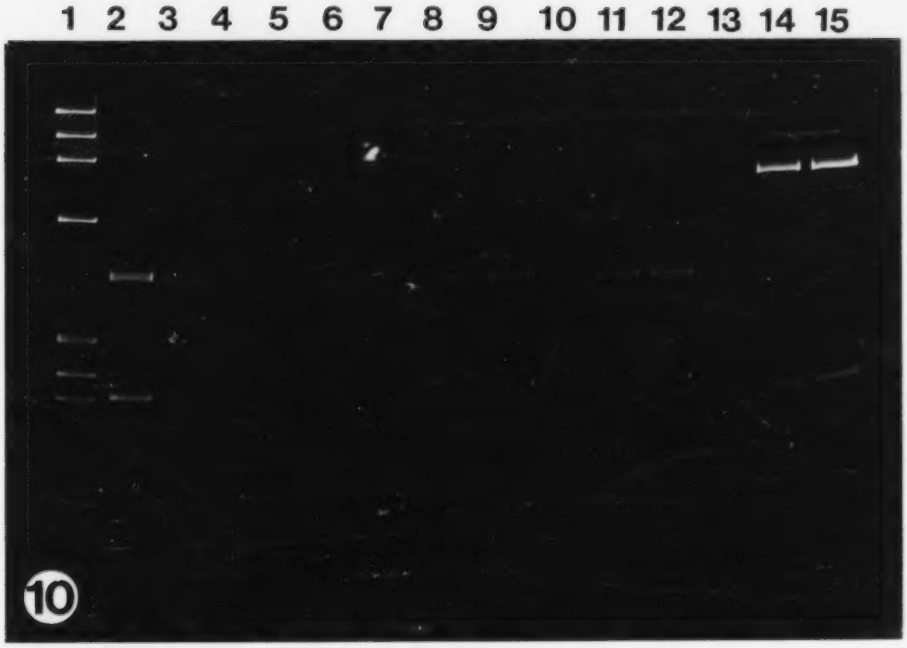
Fig. 9. RFLP analysis of products of DNA amplification primed with universal primer pair R16F2/R2. Amplified DNA was digested with *Kpn* I; legend for lanes from 1 to 16 is the same as for Fig. 8.

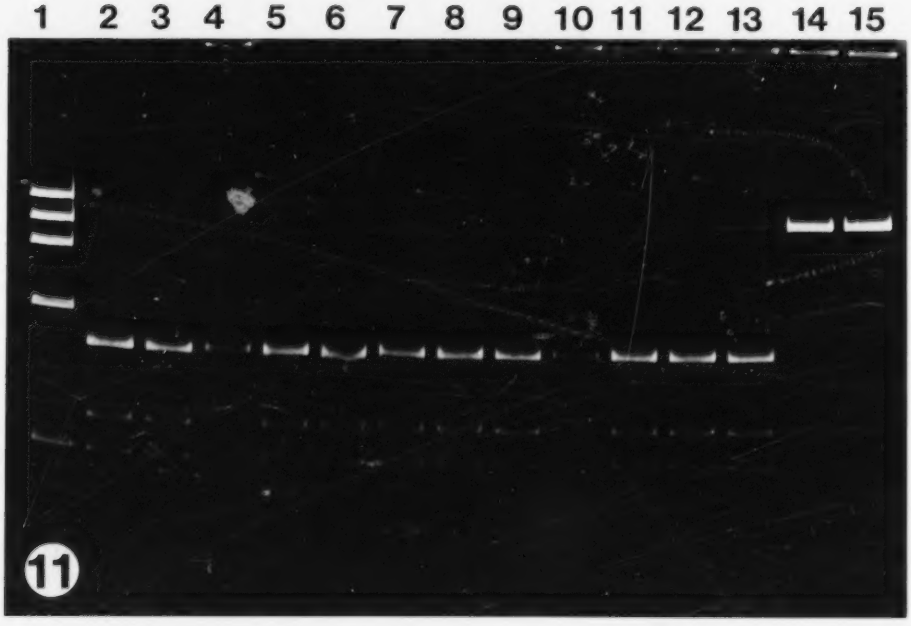

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
8

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
9

Fig. 10. RFLP analysis of products from amplification of the phytoplasma 16S rRNA gene sequence primed by the group-specific oligonucleotide pair R16F1/R1 using *Alu* I. Lane 1, ØX174 RF I DNA *Hae* III digest; lanes from 2 to 15: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, SpAY, SAY, FAY, TS, FBP and TBB.

Fig. 11. RFLP analysis of PCR products primed by primer pair R16F1/R1 using *Kpn* I restriction endonuclease; legend for lanes from 1 to 15 is the same as for Fig. 10.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
10


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
11

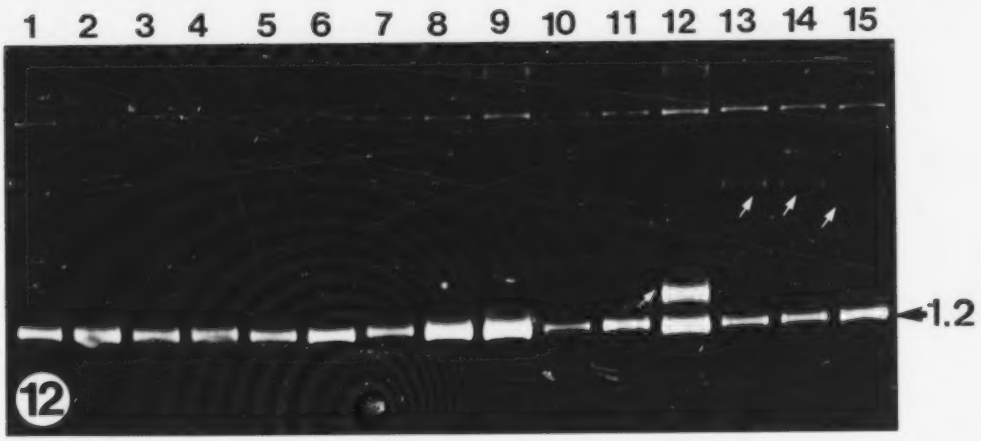
Digestions of PCR products obtained with the group-specific primer pair R16F1/R1 resulted in two collective RFLP pattern types (Fig. 10 and 11). It was not possible to differentiate between TS and other AY isolates. The restriction pattern of TBB and FBP (lanes 14 and 15) was the same, but different from other AY isolates.

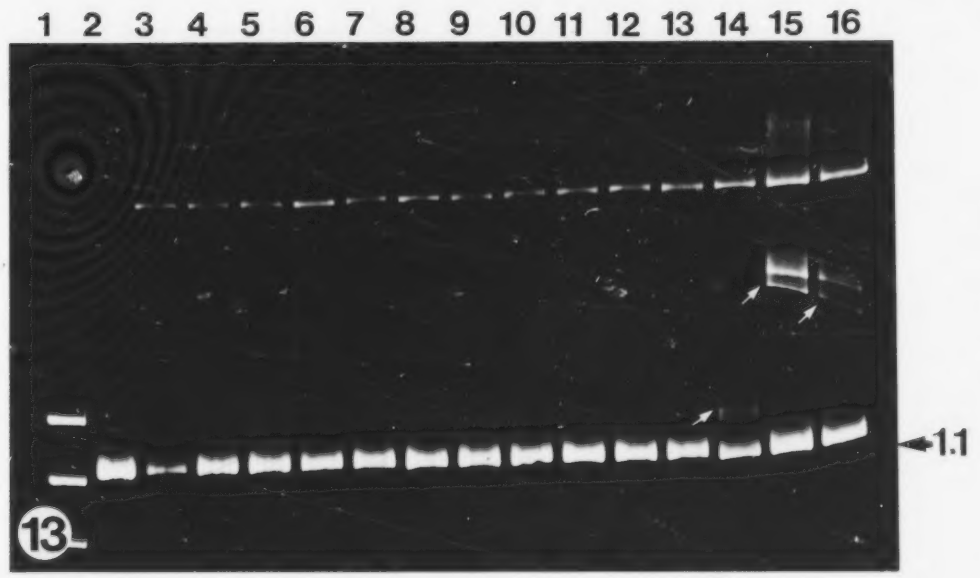
**Heteroduplex mobility analysis**

The corresponding fragments (1.2 kb or 1.1 kb) of 16S rDNA were amplified using universal or group-specific primer pair from a number of phytoplasma isolates, which represent a variety of epidemiological origins. Heteroduplexes were formed by denaturing and reannealing mixtures of amplified DNA fragments from divergent phytoplasma isolates. Pairwise combination of amplified products resulted in formation of heteroduplexes between AY27 (used as a standard) and several other isolates (Fig.12, lanes 12, 13, 14 and 15; and Fig. 13, lanes 14, 15 and 16). The largest mobility shift and divergence was observed for heteroduplexes formed with CP and PWB (Fig.12, lanes 13 and 14); intermediate shifts and divergence were observed for heteroduplexes formed with TBB and FBP (Fig.12, lane 15; and Fig.13, lanes 15 and 16); and the smallest shift and divergence was observed with TS phytoplasma isolate (Fig. 12, lane 12; and Fig. 13, lane 14). Each possible heteroduplex is formed and the strand-specific composition of mismatched and unpaired nucleotides (gaps) affects their mobility through the gel matrix. A phylogenetic tree based on HMA shows genetic relationship between different phytoplasma isolates (Fig. 17).

Fig. 12. Polyacrylamide gel showing the range of heteroduplex mobilities (indicated by arrows) determined with different phytoplasma isolates. PCR products were obtained after amplification with universal primer pair. A PCR product from AY27 strain was used as an internal standard and it was mixed with amplified DNA from each of the phytoplasma isolates. Heteroduplexes were formed between AY27 and TS, PWB, CP and TBB.; lanes from 1 to 15: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, FAY, TS, PWB, CP and TBB.

Fig. 13. Ethidium bromide-stained polyacrylamide gel showing the range of heteroduplex mobilities (indicated by arrows) determined with various phytoplasma isolates. PCR products obtained with group-specific primer pairs were used. Lane 1, ØX174 RF I; lanes from 2 to 16: AY27, WAY, EAY, FrAY, FHAY, BHAY, PMAY, WEAY, SpAY, SAY, FAY, TS, TBB and FBP.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.2
12


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
1.1
13

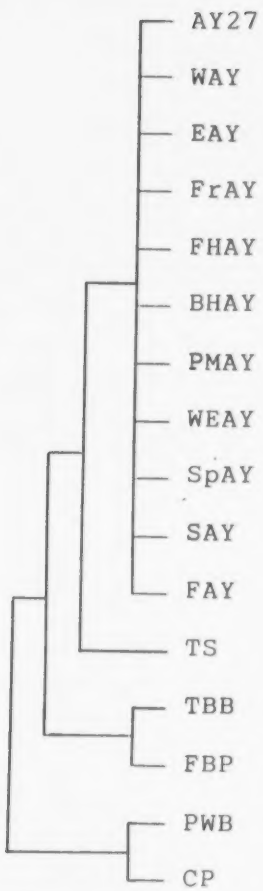



Fig. 17. Cladogram of DNA distances inferred from heteroduplex mobility analysis of 16 phytoplasmas 16S rDNA. Lateral distances are not taken into consideration.

**Temperature gradient gel electrophoresis**

TGGE was applied to detect a polymorphic DNA and to compare its result with those obtained by HMA. First, amplified DNA from standard phytoplasma isolate (AY27) was analyzed by TGGE (Fig.14). The results of the analyses of renatured PCR mixtures (AY27+PMAY and AY27+TS) are shown in Figs. 15 and 16. After denaturation and renaturation of PCR mixture from AY27 and TS , the heteroduplex specific for this combination was formed (indicated by arrow). The homoduplex has the same size as amplified DNA fragments (1.2 Kb). The homoduplex exerts the highest thermal stability because the heteroduplex was less stable due to the mismatch formation.

Fig. 14. TGGE of PCR product (1.2 Kb) from AY27 phytoplasma isolate used as an internal standard in this analysis.

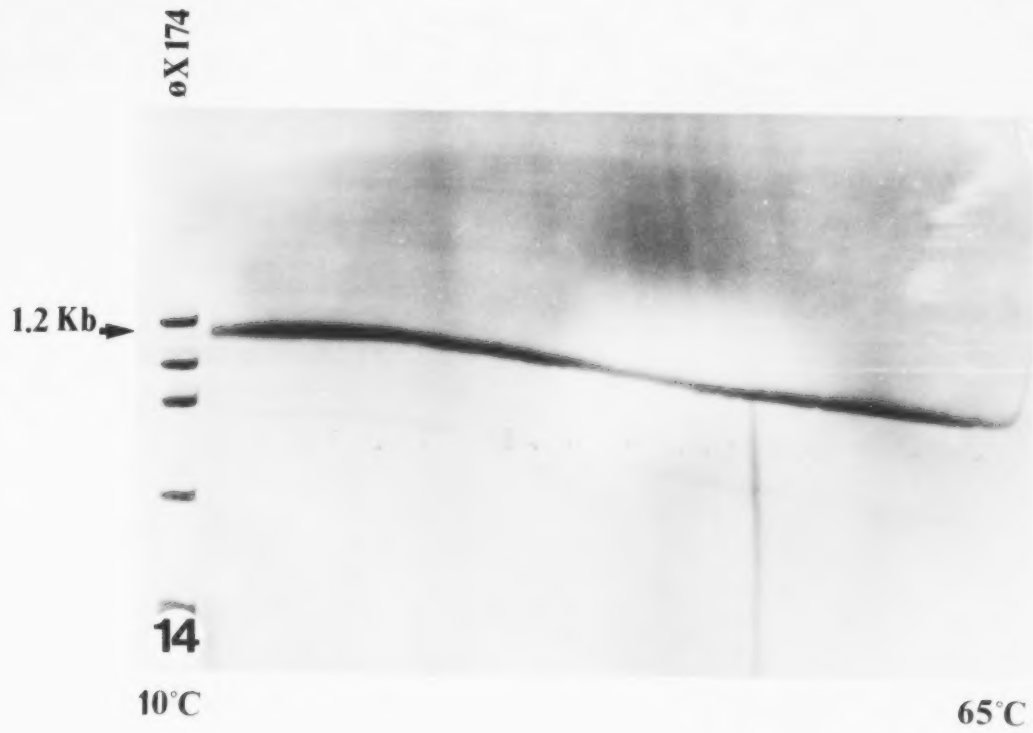
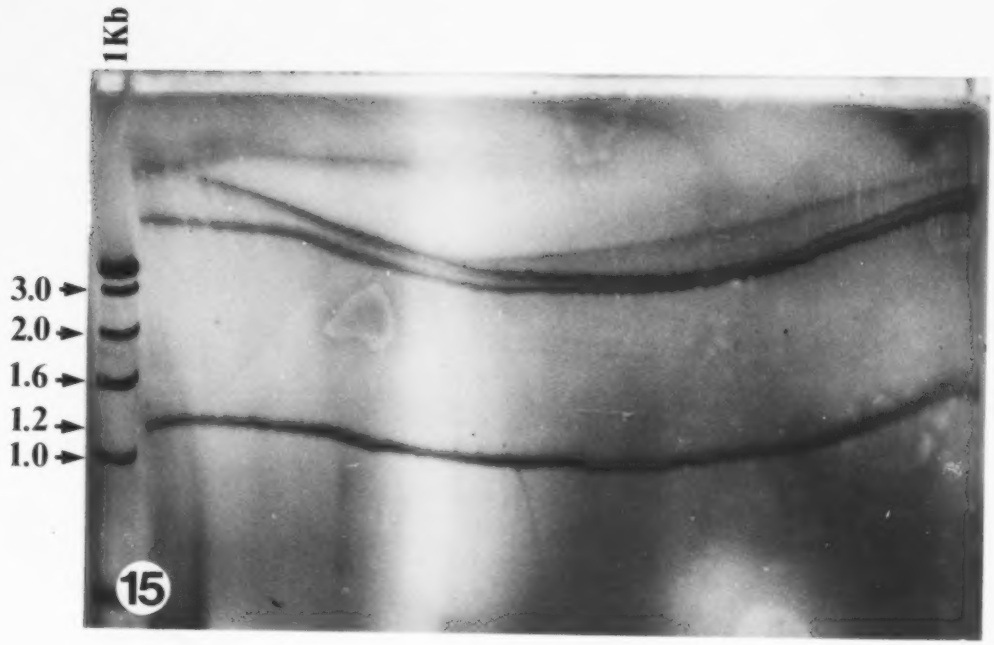

øX174
1.2 Kb
14
10°C
65°C

Fig. 15. TGGE of PCR products from two AY isolates, AY27 and PMAY after denaturation/renaturation cycle.

Fig. 16. TGGE of renatured PCR mixture from AY27 and TS. In a denaturation/renaturation cycle heteroduplex was formed and easily observed (indicated by arrow) in perpendicular TGGE.



10°C 65°C

# Discussion

The recent development of PCR-based assays provides a convenient means of obtaining 16S rRNA genes from phytoplasmas and other mollicutes without removing eukaryotic and organelle genomic DNAs when specific primers are available and thermocycling conditions are properly controlled. PCR assays using primer pairs of the sequence of cloned EY, AY, or other phytoplama DNA fragments have been employed effectively to detect ET, AY, or other phytoplasma strains associated with affected plant hosts (Deng and Hiruki, 1991b; Davis *et al.*, 1992; Lee *et al.*, 1992; Schaff *et al.*, 1992). However, such primers may not amplify DNA of all strains of a phytoplasma.

The present study also demonstrated that a PCR-based procedure using a "universal" primer pair designed on the basis of 16S rDNA sequence from a strain of Michigan AY phytoplasma (MIAY-86-7) by Lee *et al.* (1993b) can be employed effectively to detect and identify a broad array of known and unknown phytoplamas from various host plants. Enrichment of phytoplasma DNA also improved PCR amplification of 16S rRNA genes. By employing "universal" primer pair in PCR amplification, phytoplasmas16S rDNA sequence were amplified, with one exception, from nucleic acid samples extracted from all 16 phytoplasma-infected plants. PCR with template nucleic acid extracted from dill (*Anethum graveolens* L.) yielded no observable DNA amplification. It is possible that DNA template of this aromatic plants contained inhibitors of PCR reaction or symptomatic plants had insufficient phytoplasma DNA templates

capable of being amplified in the present PCR experiments. No PCR products were obtained from samples extracted from healthy plants.

The clear advantage of non-specific primers is that one pair of primers will suffice to provide a yes or no answer to presence of phytoplasma agent. However, there are several disadvantages to the use of such "universal" primers: **(1)** some Gram-positive bacteria, related phylogenetically to phytoplasmas, may yield an amplification product with some of the primers. The careful selection of primers may overcome this difficulty. **(2)** In plant material, chloroplast 16S rRNA genes may react with these primers. **(3)** Furthermore, "universal" primer pairs are not applicable for epidemiological studies where more than one type of phytoplasma is associated with a given disease.

Recently the knowledge of the conserved and non coserved regions of the complete sequence of the 16S rRNA gene has been used for preparing of specific primers from these gene (Firrao *et al.*, 1993; Lee *et al.*, 1994). These specific primers may also be utilized for diagnostic purposes avoiding the second step of RFLP after PCR amplification. Our study demonstrated that a given phytoplasma group-specific primer pair R16F1/R1 can be employed to prime specific amplification of phytoplasma 16S rDNA sequences of interest. Absence of PCR products after DNA amplification from PWB and CP confirmed the previous results (Deng and Hiruki, 1991b) that those two isolates belong to a geneticaly distinct phytoplasma group.

In both sets of primers, phytoplasma fragments were equally amplified in the range from 1 μg to 100 pg of total sample DNA

extracted from infected leaves.

Further differentiation among studied phytoplasma isolates was achieved by RFLP analyses of the amplified phytoplasma 16S rDNA sequences using two selected restriction enzymes. *Alu* I and *Kpn* I restriction enzymes were shown to be particularly useful to differentiate the 16SrI group (AY group I) from other phytoplasma groups. RFLP analysis with the amplification products revealed a genetic diversity among the phytoplasmas examined. It was possible to differentiate between TS and other AY phytoplasma isolates only when the PCR product obtained by the "universal" primer pair was used for restriction analysis. Restriction patterns of FBP and TBB were the same, but different from CP, PWB and all other phytoplasma isolates. Although DNA from FBP and TBB phytoplasma isolates were also amplified with the specific primer pair, they had a completely different restriction pattern in relation to other AY isolates. This result was not consistent with those previously published (Davis *et al.* 1990b and Lee *et al.*, 1993b). The TBB isolate used in their reserch was originally from Arkansas and had the same restriction pattern as other members of the AY phytoplasma cluster I (e.g., AY27, EAY, WAY). The TBB phytoplasma isolate included in our study was from Australia and based on the restriction pattern result it can not be placed in the same AY cluster. RFLP analyses of phytoplasma 16S rDNA sequences were successfully employed as a means to assess the phylogenetic relationships among various phytoplasmas.

In the present work, we have also investigated the use of DNA HMA was used to differentiate among various phytoplasma isolates.

The results indicated that HMA provides a simple screening tool for determining relationships between phytoplasma isolates. By the formation of the heteroduplexes even a very low degree of genetic diversity was detected, as was the case with TS isolate. All the results from HMA were in agreement with those achieved by PCR and especially RFLP analyses. The molecular size of most heteroduplexes was significantly higher than that of homoduplexes, which was actually the same as the size of PCR products (1.2 kb). The band of heteroduplex could be located easily in the position expected on the gel, because even a single 3-base pair gap can induce a noticeable mobility retardation in heteroduplexes formed (Delwart *et al.*, 1993). The degree of mobility retardations of heteroduplexes in polyacrylamide gels were in proportion with the degree of genetic divergence between analyzed phytoplasma isolates. The greater reduction in mobility caused by gaps relative to mismatched nucleotides probably reflected the sharp bend in the double-stranded DNA molecule required to accommodate the extra nucleotides and the resulting impairment of migration through the gel matrix. Based on the DNA distances between heteroduplexes and homoduplexes derived from HMA, the phylogenetic relationships among all studied phytoplasma isolates have been determined. Experiments with PCR products obtained by amplification with "universal" and group-specific primer pairs appeared to give the same results in HMA.

A major advantage of HMA resides in the high accuracy of the procedure to differentiate among different phytoplasma isolates. RFLP-based phytoplasma differentiation used different restriction enzymes to screen differences among phytoplasma-DNA sequences,

the prerequisite for RFLP analysis being that the enzyme-recognition sequence should exist in the phytoplasma DNA sequence. HMA is performed on the basis that DNA heteroduplexes formed between related sequences have a reduced mobility in polyacrylamide gels proportional to their degree of divergence. Our result suggest that HMA will prove important in phytoplasma differentiation when other methods, e.g. RFLP, are not readily applicable to differentiate between very close phytoplasmas.

TGGE may be applied to resolve many different questions, but in this study it is emphasized that TGGE may be applied as an additional or a separate method to detect and analyse polymorphic phytoplasma DNA. Results obtained by gradient gel electrophoresis supported the results from HMA. AY27, used as a internal standard, was first analysed separately, then as a mixture with PMAY, as a member of the AY group, and TS phytoplasma isolate after a denaturation/renaturation cycle. TS phytoplasma isolate was chosen for TGGE analysis as this isolate was the most difficult one to differentiate among other phytoplasma isolates belonging to the AY group. A heteroduplex formed in the AY27/TS combination was separated by temperature gradient and easily identified. As it was expected, a combination of AY27 and PMAY did not result in the formation of a specific heteroduplex.

Phytoplasmas are associated with both plant and insect vectors. It has long been suspected that a host plant or insect can be infected by more than one type of phytoplasma. Mixed infections by a primary and secondary phytoplasma(s) can occur (Lee *et al.*, 1994), although the secondary phytoplasma(s) is usually present at very low titer.

To meet with various requirements in the diagnosis of phytoplasma diseases, there are constant needs for further improvements of existing procedures and for the development of new techniques (Hiruki, 1988). It is now recognized that a genomic group of AY phytoplasma-affiliated strains can be identified by DNA-based methods (Davis *et al*, 1992, Lee *et al.*, 1992; Davis and Lee, 1993; Lee *et al.*, 1994). The AY phytoplasma group contains a variety of phytoplasma strains, including some previously termed AY phytoplasmas and others that have been given different names (Lee *et al.*, 1992).

In this study, it was possible to detect and identify several new phytoplasma isolates (PMAY, SpAY, SAY, FAY and WEAY) from plants growing naturally in the field, which were classified as being the members of the AY group by all the methods used. It was also very important to compare different molecular techniques; to assess how sensitive, fast, reliable and expensive they are and which one can be mostly recommended for this kind of research. Based on the results obtained, HMA, combined with PCR, has been shown to be a vary simple, fast, and reliable method for detection and differentiation among different strains and groups of phytoplasmas and thus highly recommendable.

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# Bibliography


**Ahrens, U. and Seemüller, E. 1992.** Detection of DNA of plant pathogenic Mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA Gene. Phytopathology 82: 828-832.

**Arora, Y.K. and Sinha, R.C. 1988.** Plant pathogenic mycoplasmas: Morphological and biochemical characteristics. pp. 3-27 In: Mycoplasma diseases of crops. Maramorosch, K. and Raychaudhuri, S.P. (eds.), Springer-Verlag. New York Inc.

**Barros, F., Muñoz-Barús, I., Lareu, M.V., Rodriguez-Calvo, M.S. and Carracedo, A. 1994.** Double- and single-strand conformation polymorphysm analysis of point mutations and short tandem repeats. Electrophoresis 15: 566-571.

**Bernet, C., garret, M., De Barbeyarac, B., Bebear, C. and Bonnet, J. 1989.** Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. J. Clinical Microbiol. 27: 2492-2496.

**Bertaccini, A., Davis, R.E., Lee, I.M., Conti, M., Dally, E.L. and Douglas, S.M. 1990** Detection of chrysanthemum yellows mycoplasmalike organism by dot hybridization and Southern blot analysis. Plant Disease 74: 40-43.

**Bertaccini, A., Davis, R.E., Hammond, R.W., Vibio, M., Bellardi, M.G. and Lee, I.-M. 1992.** Sensitive detection of mycoplasmalike organisms in field-collected and *in vitro* propagated plants of *Brassica*, *Hydrangea* and *Chrysanthemum* by polymerase chain reaction. Ann. Appl. Biol. 119: 593-599.

**Black, L.M. 1940.** Mechanical transmission of aster-yellows virus to leaf hoppers. Phytopathology 30: 2.

**Black, L.M. 1943.** Some properties of aster-yellows virus. Phytopathology 33: 2.

**Blanchard, A., Gautier, M. and Mayau, V. 1991.** Detection and identification of mycoplasmas by amplification of rDNA. FEMS Microbiol. Lett. 81: 37-42.

**Brock, T.D. and Freeze, H. 1969.** *Thermus aquaticus* gen.n. and sp.n. a non-C sporulating extreme thermophile. J. Bacteriol 98: 289-297.

**Caudwell, A., Meignoz, R., Kuszala, C., Larrue, J., Fleury, A., and Boudon, E. 1982.** Purification serologique et observation ultra-microscopique de l'agent pathogene (MLO) de la

flavescence doree de la vigne dans lesextrains liquides de feves (*Vicia faba)* malades. C.R.Soc. Biol. 176: 723-729.

**Ceranic-Zagorac, P and Hiruki, C. 1994.** Detection of Canadian aster yellows isolates by PCR. Proceedings of the 15th Annual Meeting of Plant Pathology of Alberta, p. 7 (abstract).

**Chang, K.F., Hwang, S.F. and Mirza, M. 1993.** Symptomathology and the occurance of mycoplasma-like organisms diesease of statice (*Limonium sinuatum*) in Alberta, Canada. J. Plant Dis. Protection 101: 113-123.

**Chen, M.H. and Hiruki, C. 1975.** Electron microscopy of mycoplasma-like bodies associated with clover proliferation disease. Proc. Am. Phytopathol. Soc. 2: 52 (abstract).

**Chen, M.H. and Hiruki, C. 1977**. Efect of dark treatment on the ultrastructure of aster yellows agent *in situ*. Phytopathology 67: 321-324.

**Chen, M.H. and Hiruki, C. 1978.** Occurrence of tubular structures in *Vinca rosea* infected with the Alberta isolate of the aster yellows agent. Protoplasma 95: 207-216.

**Chen, J., Chang, C.J., Jarret, R. and Gawel, N. 1990.** Cloning of DNA fragments isolated from a mycoplasma-like organism (MLO) associated with walnut witches'-broom disease, *Proceedings of the 8th International Congress of the international Organization for Mycoplasmology*, 1: 227-228.

**Chen, T.-A., Lei, J.D., and Lin, C.P. 1992.** Detection and `identification of plant and insect mollicutes. pp. 393-424 In The Mycoplasmas, Vol. 5 R.F. Whitcomb and J.G. Tully, (eds.), Academic Press, New York.

**Chiykowski, L.N. 1962**. Clover phyllody virus in Canada and its transmission. Can. J. Bot.40: 397-404.

**Chiykowski, L.N. 1971.** Interaction between clover phyllody and celery-infecting aster yellows causal agents in plants. Can. J. Bot. 49: 233-235.

**Chiykowski, L.N. and Sinha, R,C. 1990.** Differentiation of MLO diseases by means of symptomatology and vector transmission. Zbl. Suppl. 20 Gustav Fischer Verlag Stuttgard, New York, pp: 280-287.

**Dale, J.L. and Kim, K.S. 1969.** Mycoplasma bodies in dodder parasitizing aster yellows-infected plants. Phytopathology 59, 1765-66.

**Davis, M.J., Konai, M. and Bak, W.-C. 1990.** Electrophoretic separation of chromosomal DNA of mycoplasma-like organisms. IOM Letters, the 8th IOM Congress, 1: 231-232 (abstract).

**Davis, M.J., Tsai, J.H., Cox, R.L., McDaniel, L.L. and Harrison, N.A. 1987.** DNA probes for detecting the maize bushy stunt mycoplasma-like organisms (MBS-MLO). Phytopathology 77: 1769 (abstract).

**Davis, M.J., Tsai, J.H., Cox, R.L., McDaniel, L.L. and Harrison, N.A. 1988.** Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol. Plant-Microbe Intract. 1: 295-302.

**Davis, R.E., Dally, E.L., Bertaccini, A., Lee, I.-M., Credi, R., Osler, R., Savino, V., Carraro, L., Di Terlizzi, B., and Barba, M. 1993.** Restriction fragment length polymorphism analyses and dot hybridizations distinguish mycoplasmalike organisms associated with *flavescence dorée* and southern European grapevine yellows disease in Italy. Phytopathology 83: 772-776.

**Davis, R.E. and Lee, I.-M. 1993.** Cluster-specific polymerase chain reaction ampliofification of 16S rDNA sequences for detection and identification of mycoplasmalike organisms. Phytopathology 83: 772-776.

**Davis, R.E., Sinclair, W.A., Lee, I.-M. and Dally, E.L. 1992.** Cloned DNA probes specific for detection of a mycoplasmalike organism associated with ash yellows. Mol. Plant-Microbe Interact. 5: 163-169.

**Davis, R.E., Whitcomb, R.F. and Steere, R.L. 1968.** Remission of aster yellows by antibiotics. Science 161: 793-795.

**Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983.** A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1: 19-21.

**Delwart, E.L., Shpaer, E.G., Louwagie, J., McCutchan, F.E., Grez, M., Rübsamen-Weigmann, H. and Mullins, J.I. 1993.** Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 *env* genes. Science 262: 1257-1261.

**Denes, A.S.J. and Sinha, R,C. 1991.** Extrachromosomal DNA elements of plant pathogenic mycoplasmalike organisms. Can. J. Plant. Path. 13: 26-32.

**Deng, S.J. 1991.** Molecular diagnosis and characterization of mycoplasma-like organisms associated with clover proliferetion. Ph.D. Thesis. 197pp., University of Alberta, Edmonton, Canada.

**Deng, S.J. and Hiruki, C. 1990a.** Enhanced detection of a plant pathogenic mycoplasma-like organism by polymerase chain reaction. Proc. Japan. Acad., 66B: 140-144.

**Deng, S.J. and Hiruki, C. 1990b.** Molecular cloning and detection

of DNA of the mycoplasmalike organisms associated with clover proliferation. Can. J. Plant Pathol. 12: 383-388.

**Deng, S.J. and Hiruki, C. 1991a.** Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. J. Microbiol. Methods 14: 53-61.

**Deng, S.J. and Hiruki, C. 1991b.** Genetic relatedness between two nonculturable mycoplasmalike organisms revealed by nucleic acid hybridization and polymerase chain reaction. Phytopathology 81: 1475-1479.

**Deng, S.J, Hiruki, C., Robertson, J.A. amd Stemke, G.W. 1992.** Detection by PCR and differentiation by restriction fragment length polymorphysm of *Acholeplasma*, *Spiroplasma*, *Mycoplasma* and *Ureaplasma*, based upon 16S rRNA genes. PCR Methods Appl. 1: 202-204.

**Doi, Y., Teranaka, M., Yora, K., and Asuyama, H. 1967.** Mycoplasma or PTL group-like microorganisms found within the phloem elements of plant infected with mulberry dwarf, potato witches'-broom, aster yellows or Paulowinia witches'-broom. Ann. Phytopathol. Soc. Japan 33: 259-266.

**Doyle, J.J. and Doyle, J.L. 1990.** Isolation of plant DNA from fresh tissue. Focus (Life Technol. Inc.) 12: 13-15.

**Firrao, G., Gobbi, E. and Locci, R. 1993.** Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmalike organisms. Phytopathology 83: 602-607.

**Florence, E.R. and Cameron, H.R. 1978.** Three-dimensional structure and morphology of mycoplasma-like bodies associated with albino disease of *Prunus avium*. Phytopathology 68: 75-80.

**Freitag, J.H. 1964.** Interaction and mutual suppression among three strains of aster yellows virus. Virology 24: 401-413.

**Freitag, J.H. 1967.** Interaction between strains of aster yellows virus in the six spotted leafhopper *Macrosteles fascifrons*. Phytopathology 57: 1016-1024.

**Frydenberg, J. and Christiansen, C. 1985.** The sequence of 16S rRNA from mycoplasma strain PG50. DNA 4: 127-137.

**Garnier, M., Martin-Gros, G., Iskra, M.L., Zreik, L., Fos, A. and Bové, J.M. 1990.** Monoclonal antibodies against the MLO associated with tomato stolbur and clover phyllody. pp. 263-269 In: Proc. Conf. Int. Organ. Mycoplasmol. 7th. Zentralbl. Bakteriol. Mikrobiol. Hyg., Ser. A.

**Golino, D.A., Oldfield, G.N. and Gumpf, D.J. 1987.** Transmission characteristics of the beet leafhopper transmitted virescence agent. Phytopathology: 77: 954-957.

**Golino, G.A., Oldfield, G.N. and Gumpf, D.J. 1988.** Induction of flowering through infection by the beet leafhopper transmitted virescence agent. Phytopathology 78: 285-288.

**Ghosh, B.K., Misra, S. and Muniyappa, V. 1988**. Comparative morphology of mycoplama-like organisms. pp. 141-159 In: Mycoplasma diseases of crops. K. Maramorosch and S.P. Raychaudhurei (eds.) Springer-Verlag. New York Inc.

**Granados, R.R. and Chapman, R.K. 1968.** Identification of some new aster yellows virus strains and their transmission by the aster leafhopper, *Macrosteles fascifrons*. Phytopathology 58: 1685-1692.

**Granados, R.R., Maramorosch, K. and Shikata, E. 1968.** Mycoplasma: suspected etiologic agent of corn stunt. Proc. Nat. Acad. Sci. U.S. 60, 841-844.

**Griffiths, H.M., Sinclair, W.A., Davis, R.E., Lee, I.-M., Dally, E.L., Guo, Y.-H., Chen, T.A., and Hibben, C.R. 1994.** Characterization of mycoplasmalike organisms from *Fraxinus*, *Syringa*, and associated plants from geographycally diverse sites. Phytopathology 84: 119-126.

**Harasawa, R., Mizusawa, H., Nozawa, K., Nakagawa, T., Asada, K. and Kato, I. 1993.** Detection and tentative identification of dominant mycoplasma species in cell cultures by restriction analysis of the intergenic 16S-23S rRNA intergenic spacer regions. Res. Microbiol. (Institut Pasteur) 144: 489-493.

**Hiruki, C. and Chen, M.H. 1984.** Plant mycoplasma diseases occurring in Alberta. Proc. 7th IUFRO Mycoplasma Conf., p. 7 (abstract).

**Hiruki, C., ed., 1988.** Tree mycoplasmas and mycoplasma diseases. 245 p. University of Alberta Press, Edmonton, Alberta.

**Impraim, C.C., Saiki, R.K., Erlich, H.A. and Teplitz, R.L. 1987.** Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. Biochem. Biophys Res. Commun. 142: 710-716.

**Ishiie, T., Doi, Y., Yora, K., and Asuyama, H. 1967**. Suppresive efectts of antibiotics of tetracycline group on the symptom development in mulberry dwarf disease. Ann. Phytopathol. Soc. Japan 33: 267-275.

**Jeffreys, A.J., Wilson, V., Neumann, R., Keyte, J. 1988.** Amplification of human minisatellites by the PCR: Towards DNA fingerprinting of single cells. Nucl. Acids Res. 16: 10953-10971.

**Jensen, D.D. 1959.** A plant virus lethal to its insect vector Virology 8, 164-175.

**Jensen, J.S., Søndergård-Andersen, J., Uldum, S.A. and Lind, K. 1989.** Detection of *Mycoplasma pneumoniae* in simulated clinical samples by polymerase chain reaction. Acta Pathol. Microbiol. Immunol. Scand. 97: 1046-8.

**Kirkpatrick, B.C. 1989.** Strategies for characterizing plant pathogenic mycoplasma-like organisms and their effects on plants. In: Plant-Microbe Interactions. Molecular and Genetic Perspectives. T. Kosuge and E.W. Nester (eds.), McGrawhill publishing.

**Kirkpatrick, B.C. 1991**. Mycoplasma-like organisms. Plant and invertebrate pathogens. In: the Prokaryotes, vol. 2. Edited by A. Balows, G.H. Truper, M. Dworkin, W. Harder and K.H. Schliefer, eds. New York: Springer.

**Kirkpatrick, B.C. and Fraser, J.D. 1989.** Detection and differentiation of mycoplasma-like organisms using MLO-specific ribosomal RNA oligonucleotide sequences. Phytopathology 79: 1138 (abstract).

**Kirkpatrick, B.C. and Garrot, D.G. 1984.** Detection of western X-disease mycoplasma-like organisms by enzyme-linked immunosorbent assay. Phytopathology 74: 825 (abstract).

**Kirkpatrick, B.C., Smart, C., Blomquist, C., Guerra, L., Harrison, N., Ahrens, U., Lorenz, K.-H., Schneider, B. and Seemüller, E. 1994b.** Identification of MLO strain-specific PCR primers obtained from 16/23S rRNA spacer sequences. IOM Letters, Vol. 3, p. 261 (abstract).

**Kirkpatrick, B.C., Smart, C., Gardner, S., Gao, J.-L., Shrens, U., Maurer, R., Schneider, B., Lorenz, K.-H., Seemüller, E., Harrison, N., Namba, S. and Daire, X. 1994a.** Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rRNA spacer sequences. IOM Letters, Vol. 3, p.228 (abstract).

**Kirkpatrick, B.C., Stenger, D.C., Morris, T.J., and Purcell, A.H. 1987.** Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasmalike organisms. Science 238: 197-200.

**Knight, G. 1976**. Peach rosette in virus diseases and noninfectious disorders of stone fruits in North America. U.S. Dept. of Agric. Hndbk. 437: 73-75.

**Kollar A. and Seemüller, E. 1989.** Base composition of the DNA of mycoplasma-like organisms associated with various plant diseases. J. Phytopathology 127: 177-186

**Kollar, A., Seemüller, E., Bonet, F., Saillaird, S. and Bové, J.M. 1990.** Isolation of the DNA of various plant pathogenic mycoplasmalike organisms from infected plants. Phytopathology 80: 233-3237.

**Kunkel, L.O. 1926.** Studies on aster yellows. Amer. J. Bot. 13: 646-705.

**Kunkel, L.O. 1941.** Heat cure of aster yellows in periwinkles. Amer. J. Bot. 28, 761-769.

**Kunkel, L.O. 1938.** Insects in relation to disease of fruit trees and small fruits. J. of Econ. Entomol. 31: 20 (abstract).

**Kunkel, L.O. 1952.** Transmission of alfalfa witches'-broom to nonleguminous host plant by dodder, and cure in periwinkle by heat. Phytopathology 42: 27-31.

**Kunkel, L.O.1955**. Cross protection between strains of yellows-type viruses. Adv. in Virus Res. 3, 251.

**Kuske, C.R. and Kirkpatrick, B.C. 1989.** Distribution and multiplication of western aster yellows MLOs in *Catharanthus roseus*. Phytopathology 80: 958 (abstract).

**Kuske, C.R. and Kirkpatrick, B.C. 1990.** Isolation and characterization of plasmids from the western aster yellows mycoplasma-like organism. J. Bacteriol. 172: 1628-1633.

**Kuske, C.R., Kirkpatrick, B.C., Davis, M.J. and Seemüller, E. 1991.** DNA hybridization between western aster yellows mycoplasmalike organism plasmids and extrachromosomal DNA from other plant pathogenic mycoplasmalike organisms. Mol. Plant-Microbe Interact. 4: 75-80.

**Lee, P.E. and Chiykowski, L.N. 1963.** Infectivity of aster-yellows virus preparations after differential centrifugations of extracts from viruliferous leafhoppers. Virology 21, 667-69.

**Lee, I.M. and Davis, R.E. 1986.** Prospect for in vitro culture of plant pathogenic mycoplasma-like organisms. Annu. Rev. Phytopathology 24: 339-354.

**Lee, I.M. and Davis, R.E. 1988.** Detection and investigation of genetic relatedness among aster yellows and other mycoplasma-like organisms using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1: 303-310.

**Lee, I.-M., Davis, R.E. and Hiruki, C. 1991.** Genetic relatedness among clover proliferation mycoplasmalike organisms (MLOs) and other MLOs investigated by nucleiv acid hybridization and restriction fragment length polymorphysm analyses. Appl. Environ. Microbiol. 57; 3565-3569.

**Lee, I.-M., Davis, R.E., Chen, T.-A., Chiykowski, L.N.,**

**Fletcher, J., Hiruki, C., and Schaff, D.A. 1992.** A genotype-based system for identification and classification of mycoplasmalike organisms (MLOs) in the aster yellows MLO strain cluster. Phytopathology 82: 977-986.

**Lee, I.-M. and Davis, R.E. 1992**. Mycoplasmas which infect plants and insects. pp. 379-390 In: Mycoplasmas: Molecular Biology and Pathogenesis. J. Maniloff, R.N. McElhansey, L.R. Finch, and J.B. Baseman, (eds.), Amer. Soc. Microbiol., Washington, DC.

**Lee, I.-M., Davis, R.E., Sinclair, W.A., DeWitt, N.D. and Conti,M. 1993a.** Genetic relatedness of mycoplasmalike organisms detected in *Ulmus ssp.* in the United States and Italy by means of DNA probes and polymerase chain reaction. Phytopathology 83: 829-833.

**Lee, I.-M., Gundersen, D.E., Hammond, R.W. and Davis, R.E. 1994.** Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. Phytopathology 84: 559-566.

**Lee, I.-M., Hammond, R.W., Davis, R.E. and Gundersen, D.E. 1993b.** Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83: 834-842.

**Lim, P.O. and Sears, B.B. 1989.** 16S rRNA sequence indicates that plant-pathogenic mycoplasma organisms are evolutionarily distinct from animal mycoplasmas. J. Bacteriol. 171: 5901-5906.

**Lim, P.O and Sears, B.B. 1991.** The genome size of a plant-pathogenic mycoplasmalike organism resembles those of animal mycoplasmas. J. Bacteriol. 173: 2128-2130.

**Lin, C.P and Chen, T.A., 1985.** Monoclonal antibodies against the aster yellows agent. Science 227: 1233-1235.

**Lin C.P. and Chen, T.A. 1986.** Comparison of monoclonal antibodies and polyclonal antibodies in detection of aster yellows mycoplasma-like organism. Phytopathology 76: 45-50.

**Maniatis, T.I., Fritsch, E.F. and Sambrook, J. 1982**. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

**Maramorosch, K. 1952.** Direct evidence for multiplication of aster-yellows virus in its insect vector. Phytopathology 42, 59-64.

**Maramorosch, K. 1974.** Mycoplasmas and ricketsiae in relation to plant diseases Annu. Rev. Microbiol. 28: 301-324.

**Maramorosch, K., Granados, R.R., and Hirumi, H. 1970**.

Mycoplasma diseases of plants and isects. Adv. Virus Res. 16: 135-193.

**Maramorosch, K. and Jensen, D.D. 1963.** Harmful and beneficial effects of plant viruses in insects. Annu. Rev. Microbiol. 17, 495-530.

**Maramorosch, K., Shikata, E. and Granados, R.R. 1968.** Mycoplasma-like bodies in leafhoppers and diseased plants Phytopathology 58, 886 (abstract).

**Maramorosch, K., Granados, R.R., and Hirumi, H. 1970.** Mycoplasma diseases of plants and isects. Adv. Virus Res. 16: 135-193.

**Maramorosch, K. and Raychaudhuri, S.P. Eds.1981.** Mycoplasma diseases of trees and shurbs, Academic Press, New York.

**Markham, P.G. and Towsend, R. 1979.** Experimantal vectors of Spiroplasmas, pp. 413-445 In: Leafhopper vectors and plant disease agents. K.Maramorosch and K.F.Harris, (eds.), Academic Press, New York.

**Matteoni, J.A. and Sinclair, W.A. 1989.** A note on the presence of elm yellows in the Niagara Peninsula. Phytoprotection 70: 137-1239.

**McCoy, R.E. 1983**. Wall-free prokaryotes of plants and invertebrates. pp. 2238-2246 In: The Prokaryotes, Vol. 2, M.P. Starr, H. Stolp, H.G.Truper, A. Balows and H.G. Schlegel, (eds.), Springer-Verlag, New York Inc.

**McCoy, R.E., Caudwell, A., Chang, C.J., Chen, T.A., Chiykowski, L.N., Cousin, M.T. Dale de Leeuw, G.T.N., Golino, D.A., Hackett, K.J., Kirkpatrick, B.C., Marwitz, R., Petzold, H., Shina, R.H., Sugiura, M., Whitcomb, R.F., Yang, I.L., Zhu, B.M., and Seemüller, E. 1989.** Plant diseases associated with mycoplasmalike organisms. pp. 545-560 In :The Mycoplasmas, vol.5. R.F. Whitcomb and J.G. Tully, (eds.), Academic Press, New York.

**Mullis, K.B. and Faloona, F. 1987**. Specific dynthesis of DNA in vitro via a polymerase catalysed chain reaction. Meth. Enzymol. 155: 335-350.

**Mullis, K.B., Faloona, F., Scharf, S.J., Saiki, R.K., Horn, G.T., Erlich, H.A. 1986.** Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51: 263-273.

**Murray, M.G. and Thompson, W.F. 1980.** Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. 8: 4321-4326.

**Namba, S., Oyaizu, H., Kato, S., Iwanami, S. and Tsuchizaki, T. 1993.** Phylogenetic diversity of phytopathogenic

mycoplasmalike organisms. Int. J. Syst. Bacteriol. 43: 461-467.

**Nault, L.R. and Bradfute, O.E. 1979.** Corn stunt: involvement of a complex of leafhopper-borne pathogens, pp. 561-586 In: *Leafhopper and Plant Disease Agents* (K. Maramorosch and K.F. Harris, (eds.), Academic Press, New York.

**Nasu, S., Sugiura, M., Wakimoto, S. and Iida, T.T. 1967.** On the pathogen of rice yellow dwarf. Ann. Phytopathol. Soc. Japan 33: 343-344 (abstract).

**Neimark, H.C. and Kirkpatrick, B.C. 1991.** Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic *Mycoplasma*-like organisms. Mol. Microbiol. 7: 21-28.

**Niegel, A.J., Isai, J.H., Bourne, C.M. and Richardson, P.A. 1991.** Molecular cloning and detection of chromosomal and extrachromosomalDNA of mycoplasma organisms associated with witches'-broom disease of pigeon pea in Florida. Mol. Plant-Microbe Interact. 4: 300-307.

**Nielson, M.W. 1968.** The lefhopper vectors of phytopathogenic viruses (Homoptera, Cicadellidae) taxonomy, biology and virus transmission. Tech. Bull. U.S. Dept. Agr. 1382, 386 pp.

**Ploaie, P. and Maramorosch, K. 1969.** Electron microscopic demonstration of particles resembling mycoplasma or psittacosis-lymphogranuloma-trachoma group in plants infected with European yellows-type diseases. Phytopathology 59: 536-544.

**Prince, J.P., Davis, R.E., Wolf, T.K., Lee, I.-M., Mogen, B.D., Dally, E.L., Bertaccini, A., Credi, R. and Barba, M. 1993.** Molecular detection of diverse mycoplasmalike organisms (MLOs) associated with grapevine yellows and their classification with aster yellows, X-disease, and elm yellows MLOs. Phytopathology 83: 1130-1137.

**Razin, S. 1985.** Molecular biology and genetics of mycoplasmas (Mollicutes). Microbiol. Rev. 49: 419-455.

**Riesner, D., Steger, G., Zimmat, R., Owens, R.A., Wagenhöfer, M., Hillen, W., Vollbach, S. and Henco, K. 1989.** Temperature-gradient gel electrophoresis of nucleic acids: Analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. Electrophoresis 10: 377-389.

**Riesner, D., Steger, G., Wiese, U., Wulfert, M., Heibey, M. and Henco, K. 1992.** Temperature-gradient gel electrophoresis for the detection of polymorphic DNA and for quantitative polymerase chain reaction. Electrophoresis 13: 632-636.

**Robertson, J.A., Vekris, A., Bebear, C. and Stemke, G.W. 1993.** Polymerase chain reaction using 16S rRNA gene sequences distinquishes the two biovars of Ureaplasma urealyticum. J. Clin. Microbiol.31: 824-830.

**Rosenbaum, V. and Riesner, D. 1987.** Temperature-gradient gel electrophoresis. Thermodinamic analysis of nucleic acids and proteins in purified form and in cellular extracts. Biophys. Chem. 26: 235-246.

**Ruzicka, V., März, W., Russ, A., Fisher, E., Mondorf, W. and Groß, W. 1993.** Characterization of the gene for apolipoprotein E5-Frankfurt ($Gln^{81}$ -> Lys, $Cys^{112}$ -> Arg) by polymerase chain reaction, restriction isotyping, and temperature gradient gel electrophoresis. Electrophoresis 14: 1032-1037.

**Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S., Higuchi, R.H., Horn, H.T., Mullis, K.B. and Erlich, H.A. 1988.** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

**Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H.A. and Arnheim, N. 1985.** Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. Science 230: 1350.

**Sears, B.B. and Kirkpatrick, B.C. 1994.** Unveiling the evolutionary relationships of plant-pathogenic mycoplasmalike organisms. Amer. Soc. Microbiol. News, 60: 307-312.

**Schaff, D.A., Lee, I.-M. and Davis, R.E. 1992.** Sensitive detection and identification of mycoplasmalike organisms by polymerase chain reaction. Biochem. Biophys. Res. Comm. 186: 1503-1509.

**Sears, B.B., Lim, P.O., Holland, N., Kirkpatrick, B.C. and Klomparens, K.L. 1989.** Isolation and characterization of DNA from a mycoplasmalike organism. Mol. Plant-Microbe Interact. 2: 175-180.

**Seemüller, E., Schaper U. and Zimbelmann, F. 1984.** Seasonal variation in the colonization patterns of mycoplasma-like organisms associated with apple proliferation and pear decline. Zeit. Pfl. krank. schutz, 91: 371-382.

**Seliskar, C.E. 1976.** Mycoplasma-like organism found in the phloem of bunch-diseased walnuts. Forest Sci. 22: 144-148.

**Sinha, R.C. 1976.** Ultrastructure of mycoplasma-like organisms purified from clover phyllody affected plants. J. Ultrastruct. Res. 54: 183-189.

**Sinha, R.C. 1979.** Purification and serology of mycoplasma-like organisms from aster yellows-infected plants. Can. J. Pl.

Pathol. 1: 65-70.

**Sinha, R.C. 1983.** Relative concentration of mycoplasma-like organisms in plants various times after infection with aster yellows. Can. J. Pl. Pathol. 5: 7-10.

**Sinha, R.C. 1988.** Serological detection of mycoplasma-like organisms in plants. pp.143-156. In: Tree Mycoplasmas and Mycoplasma Diseases. C. Hiruki, (ed.), Univ. Alberta Press, p.143-156.

**Sinha, R.C. and Benhamou, N. 1983.** Detection of mycoplasma-like organism antigenes from aster yellows-diseased plants by two serological procedures. Phytopathology 73: 1199-1202.

**Smith, R.E. 1902.** Growing china asters. Hatch Experimental Station. Massachusettes Agric. College Bull. 79:1-26.

**Spann, W., Pachmann, K., Zabnienska, H., Pielmeier, A. and Emmerich, B. 1991.** In situ amplification of single copy gene segments in individual cells the polymerase chain reaction. Infection 19 : 242-244.

**Steere, R.L. 1967.** Gel filtration of aster yellows virus. Phytopathology 57: 832 (abstract).

**Swingle, R.U. 1938.** A phloem necrosis of elm. Phytopathology 28: 757-759.

**Taschke, c., Ruland, K. and Herrmann, R. 1987.** Nucleotide sequence of the 16S rRNA of *Mycoplasma hyopneumonie*. Nucl. Acids Res. 15: 3918.

**Tsai, J.H., Chen, X.-Y., Shen, C.-Y. and Jin, K.-X. 1988.** Mycoplasmas and fastidious vascular prokaryotes associated with tree diseases in China. pp. 69-96 In: Tree Mycoplasma and Mycoplasma Diseases. C. Hiruki, (ed.), Univ. Alberta Press, Alberta.

**Walbot, V. and Goldberg, R. 1979**. Plant genomic organization and its relationship to classical plant genetics. pp: 3-40 In: Nucleic acids in plants. T.C. Hall and I.W. Davies, (eds.), CRC Press, Boca Raton, FL.

**Waters, H. and Hunt, P. 1980.** The *in vivo* three-dimensional form of a plant mycoplasma-like organism by the analysis of serial ultrathin sections. J. Gen. Microbiol. 116: 111-131.

**Whitcomb, R.F. and Black, L.M. 1982.** Plant and Arthropod Mycoplasmas: A Historical Perspective. pp. 40-81 In: Plant and Insect Mycoplasma Techniques. M.J. Daniels and P.G. Markham, eds. Halster Press, New York.

**Whitcomb, R.F. and Davis, R.E. 1969.** Properties of the aster yellows agent. Phytopathology 59: 1561 (abstract).

**Whitcomb, R.F. and Davis, R.E, 1970.** Mycoplasma and

phytarboviruses as plant pathogens persistently transmitted by insects. Annu. Rev. Entomol. 15: 405-464.

**Woese, C.R. 1987.** Bacterial evolution. Microbiol. Rev. 51: 221-271.

**Worley, J.F. 1969.** Chains, filamentous forms, and budding of mycoplasmalike organisms in aster yellows-infected plants. Phytopathology 59: 1561.

**Worley, J.F. 1970.** Possible replicative forms of a mycoplasmalike organisms and their location in aster yellows diseased Nicotiana and Aster. Phytopathology 60: 284-292.

**Yoshikawa, N., Nakamura, H., Sahashi, N., Kubono, T., Katsube, K., Shoji, T. and Takahashi, T. 1994.** Amplification and nucleotide sequences of ribosomal protein and 16S rRNA genes of mycoplasma-like organism associated with paulownia witches' broom. Ann. Phytopathol. Soc. Japan 60: 569-575.

**Zeyen, R.J. and Banttari, E.E. 1969**. Electron and light microscopy of oat tissue infected with the oat blue dwarf virus. Phytopatology 59, 1059.

**Zhong, Q. and Hiruki, C. 1994.** Genetic differentiation of phytoplasma isolate determined by a DNA heteroduplex mobility assay. Proc. Japan Acad. 70B: 127-131.